REMARKS

This paper is submitted in response to the Advisory Action mailed April 7, 2005. The Response submitted March 21, 2005 and the amendments to the claims made therein were, according to the Advisory Action dated April 7, 2005, not entered because the Applicants' amendments allegedly raised new issues that would require further consideration and/or search.

This paper comprises amendments and arguments set forth in Applicants' March 21, 2005 response with some modifications, and also presents new amendments and arguments for the Examiner's consideration in view of the Request for Continued Examination submitted herewith.

Claims 1-3, 5-8, 10, 12, 14-15 and 17-19 are pending in the application. Claims 4, 9, 11, 13 16 and 17 have been canceled without prejudice to Applicants' right to pursue the canceled subject matter in other applications. Applicants request the Examiner to consider new Claims 18 and 19 which are limited to maize and, in light of the Examiner's remarks, Applicants believe to be in allowable form. Claims 18 and 19 are fully supported by the originally filed specification, and find support in the originally filed specification at, for example, page 22, line 26, to page 23, line 19. As such, no new matter has been added.

Claims 1 and 12 are currently amended to recite "…that is recalcitrant or unsuited to transformation and has a transformation efficiency of zero to 1/100….". The amended claims are fully supported by the originally filed specification at page 2, lines 3-4 and page 3, lines 3-7. Claim 7 which is dependent on Claim 1 is amended to read "….wherein the said transgene encodes a protein which ….." and does not contain new matter. Further amendments made herein do not constitute new matter.

Applicants also respectfully request consideration of a Declaration of Dr. Pascaul Perez (the "Perez Declaration") filed herewith.

THE CLAIMS ARE ENABLED

Claims 1-3, 5-6, 8, 10, 12, 15 and 17 are rejected in the Office Action mailed January 21, 2005, at pages 2-9, under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the enablement requirement because the claims allegedly "contain subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention." The Examiner has maintained the enablement rejection in the Attachment to Advisory Action mailed April 7, 2005, at *Item 11 (continued)* of the attachment.

Applicants respectfully disagree with these rejections, and submit that the pending claims, as amended, are fully enabled by the originally filed specification, drawings and claims. As a preliminary matter, Applicants respectfully request the withdrawal of rejection of the canceled Claim 17.

The Office Action dated January 21, 2005 states that the presently claimed invention is not enabled because it broadly claims applicability to different maize lines and plant types while allegedly only disclosing the use of the single maize Hi-II line (Office Action at page 3). This rejection is reiterated in the Advisory action mailed March 7, 2005.

U.S. Patent Application No. 09/ 907411 filed July 17, 2001 and published March 6, 2003 under Publication No. US2003/0046724 and U.S. Patent Application No. 10/784,418 filed February 23, 2004 and published September 30, 2004 under Publication No.

US2004/0194161, list at least 29 recipient maize genotypes that "may function in hybrid combination with Hi-II, A188 or another donor parent." Based on the documented ability of others to utilize several maize lines in generating hybrid lines, Applicants respectfully suggest that the instant invention is broadly applicable to different maize lines.

Further, Applicants note that transformation of several plant species has been achieved by utilization of T-DNA vectors. For example, various sunflower genotypes (Gurel *et al.*, Tr. J of Botany, (1999) 23:171-177), various Oilseed rape (*Brassica napus*) cultivars (Damgaard *et al.*, Transgenic Research (1997) 6: 279-288), various tomato cultivars (Davis *et al.*, Plant Cell Tissue and Organ Culture (1991) 24:115-121), canola (Stewart *et al.*, Plant Physiol (1996) 112:115-120) and melon (Bordas *et al.*, Transgenic Research (1997) 6:41-50) have been transformed using *Agrobacterium*-mediated gene transfer methods. Thus all the claimed groups of plants including crop plants, vegetables, and flowers are represented as amenable targets for *Agrobacterium*-mediated gene transfer. A person skilled in the field of plant transformation would be readily aware of lines adapted to transformation which are suitable for the type of experimental manipulation specified by the instant invention. Further, as stated by Dr. Perez in his declaration at page 2, section [4], paragraph 5, "methods of making hybrids (crossing two lines) are well known in the art for any species, thus making it possible to transform any hybrid formed from a cross between a line of interest and a line adapted to transformation."

Applicants therefore respectfully request the Examiner to withdraw the rejection under 35 U.S.C. §112, first paragraph, for allegedly lacking enablement for different maize lines and additional plant types.

Applicants in addition, respectfully direct the Examiner's attention to new Claims 18 and 19, which are limited to maize and, considering the Examiner's position, are believed to be in allowable form.

The Examiner further alleges that Applicants' arguments relating to the methods for analyzing and comparing genomes as being known in the art is not persuasive (Office Action of January 21, 2005, at pages 4-5). The Examiner reiterates cited references Welsh *et al.* (Nucleic Acids Res. (1990) 18(24): 7213-7218; hereinafter "Welsh *et al.*") and Staub *et al.* (HortScience (1996) 31(5):729-741; hereinafter "Staub *et al.*") to demonstrate the alleged unpredictability inherent in applying any marker system to a wide variety of genotypes, thereby requiring undue experimentation to determine a suitable marker system (Office Action at pages 7-8). Applicants respectfully note that Welsh *et al.* describe an arbitrarily primed polymerase chain reaction (AP-PCR) method which enables the simple and reproducible fingerprints of complex genomes without requirement of prior sequence information. Welsh *et al.*, state at page 7216, column 2, paragraph 5, that "AP-PCR will work with most genome and species" (having tested it on rice, maize and human genomes). Thus the Examiner has not explained why Welsh *et al.*, does not in fact actually exemplify the level of skill in the relevant art and, indeed, enable the discrimination of related genomes. Applicants remind the Examiner that the law on enablement allows for some experimentation. *See In re Wands*, 858 F.2d at 737 (Fed. Cir. 1988). Furthermore, U.S. Patent No. 5,332,408 relates to sequences that are specific to particular plant species, subspecies or variety (see column 4, lines 31-35), and gives methods to isolate such sequences.

The references cited *supra* clearly indicate that methods for analyzing and comparing genomes are well known in the art. The instant specification is therefore fully enabled for methods for analyzing and comparing genomes and therefore the rejection for lack of enablement should be withdrawn.

With regard to the Examiner's contention that various proteins listed in the specification are not enabled, Applicants point out that the crux of the present invention lies not on the nature of the transgene, but on plant genomic DNA exclusive of the transgene. Further, it is also noted that, from the steps of Claim 1, it is clear that the nature of the transgene is not likely to affect the claimed methods. Thus, the invention is clearly enabled for claims directed to proteins conferring agronomic or disease resistance.

Applicants also note the Examiner acknowledges that commercial elite lines are well known in the art (Office Action mailed January 21, 2005, at page 6, lines 12-13). The arguments presented to overcome the rejections of second line of interest and examples of proteins cited above, apply equally to the rejection cited by the Examiner for claims relating to commercial elite lines (*i.e.* Claim 8, which is dependent on Claim 1). Finally, Applicants reiterate that Welsh *et al.*, as well as the highly developed state of the art to which this invention is most related, evidence that the Examiner's rejection, which is based on the number of markers necessary to follow selection and alleged unpredictability of recovering the same, is unsound. In addition, the Perez Declaration at paragraphs [12] and [13], pages 5 and 6 respectively, specify the predicted number of backcrosses (between 3 or 4) to obtain a isotransgenic line of the instant invention and relative ease of determining isotransgenecity using molecular markers based on integration of the transgene only in the genome of the line of interest.

For the reasons cited above, Applicants respectfully request that the rejection of Claims 1-3, 5-8, 10, 12 and 14-15 under 35 U.S.C. §112 first paragraph, for lack of enablement be removed.

THE CLAIMS ARE SUPPORTED BY THE SPECIFICATION

Claims 1-3, 5-6, 8, 10, 12, 15 and 17 are rejected under 35 U.S.C. §112, first paragraph for allegedly failing to comply with the written description requirement. The Examiner alleges that the specification only draws reference from work done by others and does not demonstrate that the inventors had possession of the claimed invention at the time the application was filed (Office Action of January 21, 2005, at pages 8-9). Applicants respectfully disagree. As a preliminary matter Applicants respectfully request the withdrawal of rejection of canceled Claim 17.

First, the Examiner appears to be basing the written description rejections on a lack of working examples of certain embodiments of the invention. The law does not require the presence of working examples, provided that an invention is described in such a way that it can be practiced by a person skilled in the art. The present claims conform, in their language and limitations, to the specification. For reasons set forth in the preceding section and the Perez Declaration, the claims are enabled. Therefore, the Applicants were in fact in possession of the invention as claimed when this application was filed.

Second, Applicants note that the Examiner has the initial burden of presenting evidence or reasons why persons skilled in the art would not recognize in an Applicant's disclosure a description of the invention defined by the claims (541 F.2d at 265, 191 U.S.P.Q. at

98; *Ex parte Sorenson*, 3 U.S.P.Q. 2d 1462, 1463 (Bd. Pat. App. & Inter. 1987)). Applicants contend that this burden has not been met in either the June 25, 2004 Office Action or in the Office Action mailed January 21, 2005. Applicants further contend that the steps of the methods are well enunciated in the claims and in the specification. Further it has been demonstrated, using multiple references (including references cited by the Examiner, at page 18 of the Response filed October 21, 2004), that all the individual steps of the invention are well-known in the art including documents already of record demonstrating the existence of broad knowledge of these methods in the existing art at the time of filing of the invention. The claimed method is a particular combination of the known methods. Therefore a person skilled in the relevant art would readily understand that the inventors had this combination in their possession at the time of filing the application. For these reasons, Applicants respectfully request that the rejection of Claims 1-3, 5-8, 10, 12 and 14-15 under 35 U.S.C. §112 first paragraph, under the written description requirement be removed.

THE CLAIMS ARE NOT ANTICIPATED BY RAGOT

Claims 12 and 17 are rejected under 35 U.S.C. §102(b) as being anticipated by Ragot *et al.* (Techniques et utilisations des marquers moleculaires; Montpellier (France) 29-31 Mars 1994; Ed. INRA, Paris 1995 (Les Colloques, n72); pages 45-56; hereinafter "Ragot *et al*"). The Examiner alleges that Ragot *et al.*'s method could theoretically produce an isotransgenic line only containing genomic sequences from the line of interest and is therefore indistinguishable from the claimed invention (Office Action at pages 9-10). Applicants respectfully disagree, at least because theoretical assumptions cannot ground a rejection based on anticipation. As a

preliminary matter Applicants respectfully request the withdrawal of rejection of the canceled Claim 17.

Applicants assert that the basis for this rejection is improper, as it appears to arise from the Examiner's belief that the claimed subject matter cannot be achieved. The Examiner states, as part of the 102 rejection: "there is no evidence in the specification of any isotransgenic maize line that has no remaining DNA from the line suited for transformation" , and goes on to apply Ragot *et al.*, as an anticipatory reference against lines which allegedly contain residual DNA. It is improper to apply a reference as anticipatory of an alleged disclosure - anticipation needs to consider exactly what is provided by the claims. By indicating that Ragot does not achieve isogenic lines, the Examiner is essentially admitting that Ragot does not anticipate claims that provide for truly isogenic plants.

Nevertheless, Claim 12 is amended herein to read "An isotransgenic line as compared to a line of interest that is recalcitrant or unsuited to transformation and has a transformation efficiency of zero to 1/100, wherein said isotransgenic line only differs from said line of interest by the presence of the T-DNA containing the transgene." Ragot *et al.* do not specify use of a line that is recalcitrant or unsuited to transformation. Amended Claim 12 is, therefore, not anticipated by Ragot *et al*. In addition, Applicants reiterate that Ragot *et al*. clearly produce "near isogenic lines" whereas the linkage drag around the transgene would be perfectly null in the claimed invention, since the "said isotransgenic line only differs from said line of interest by the presence of the T -DNA containing the transgene" (Claim 12). Paragraph [11], page 4 of the Perez Declaration a provides a detailed description of why backcrossing to obtain a isotransgenic line, if the transgene is in the "wrong" genome of a hybrid, has a probability

approaching zero. As such, the pending claims are clearly distinct from the lines disclosed by Ragot *et al.* Applicants, therefore, respectfully request withdrawal of the rejection of Claim 12 under 35 U.S.C. §102(b).

THE CLAIMS ARE NOT OBVIOUS IN VIEW OF THE CITED ART

Claims 1-3, 5-8, 10, 12, 14-15 and 17 are rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over Ishida *et al.*, (Nature Biotech. (1996) 14:745-750; hereinafter "Ishida *et al.*") in view of Does *et al.*, (Plant Mol. Biol. (1991) 17:151-153; "hereinafter "Does *et al.*"), Hiei *et al.*, (Plant Journal (1994) 6(2):271-282; hereinafter "Hiei *et al.*"), Armstrong *et al.*, (Theoretical and Applied Genetics (1992) 84:755-762; hereinafter "Armstrong *et al.*") and Ragot *et al.* as stated for claims 1-12 and 14-16 on pages 15-18 of the previous Office Action (Office Action at pages 10-12). Applicants respectfully disagree. As a preliminary matter, Applicants respectfully request the withdrawal of rejection of the canceled Claim 17.

To establish a *prima facie* obviousness, all the claim limitations must be taught or suggested by the prior art (*In re Royka*, 490 F.2d 981, 180 U.S.P.Q. 580 (C.C.P.A. 1974). *In re Wilson*, 424 F.2d 1382, 1385, 165 U.S.P.Q. 494,496 (C.C.P.A. 1970) states that "All words in a claim must be considered in judging the patentability of that claim against the prior art." The Examiner contends that the rejection of claims is based on combinations of references while Applicants' have erroneously considered each reference individually in their October 21, 2004 response at page 23. Applicants maintain that the selection step in Claim 1 is absent and not suggested by the prior art, as discussed in Applicants' October 21, 2004 response. Applicants

assert that each cited document was not merely addressed individually, but was also addressed in the proposed combination (October 21, 2004 response at page 23).

The Examiner further contends that Applicants' statements: (1) "the claimed methods for producing isotransgenic lines require transforming a suitable line, selecting appropriate transformants, and performing backcrosses with a line of interest" (October 21, 2004 response at page 17, lines 4-6); (2) that "the present invention is directed to obtaining isotransgenic lines by combining multiple steps . . . each step being known in the art" (October 21, 2004 response at page 17, lines 18-21); and (3) "the nature of the claimed invention is a combination of known methods" (October 21, 2004 response at page 18, lines 6), render the claims obvious per Applicants' admissions. Applicants' have made no such admission and assert that the Examiner has improperly used Applicants' teachings as a blueprint to reconstruct the claimed invention bit-by-bit from disparate references that contain no motivation to combine their disclosures.

Further, Applicants invite the Examiner's attention to the Perez Declaration, which, in paragraph [14], sets forth reasons why the cited references do not render the claims obvious in view of the nature of the problem to be solved.

As such, Applicants respectfully request that the rejections under 35 U.S.C. §103(a) in view of the combination of Ishida *et al.*, Hiei *et al.*, Does *et al.*, Armstrong *et al.*, and Ragot *et al.*, be withdrawn.

CONCLUSION

Applicants believe that in light of the foregoing amendments and remarks, the claims are in condition for allowance, and accordingly, respectfully request withdrawal of the outstanding objections and rejections. The Examiner is kindly invited to contact the undersigned if helpful to advance the application to allowance, which is earnestly sought.

June 21, 2005

Respectfully submitted,

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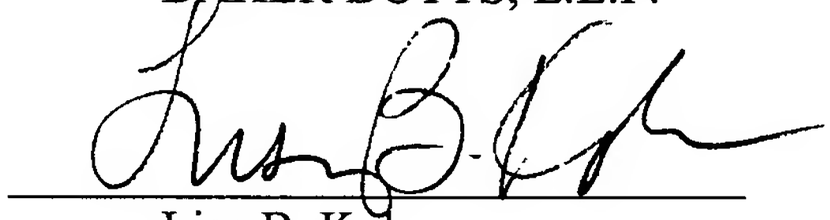

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Transfer of the yeast salt tolerance gene *HAL1* to *Cucumis melo* L. cultivars and *in vitro* evaluation of salt tolerance

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An *Agrobacterium*-mediated gene transfer method for production of transgenic melon plants has been optimized. The *HAL1* gene, an halotolerance gene isolated from yeast, was inserted in a chimaeric construct and joined to two marker genes: a selectable-neomycin phosphotransferase-II (*nptII*)-, and a reporter-β-glucuronidase (*gus*)-. The entire construct was introduced into commercial cultivars of melon. Transformants were selected for their ability to grow on media containing kanamycin. Transformation was confirmed by GUS assays, PCR analysis and Southern hybridization. Transformation efficiency depended on the cultivar, selection scheme used and the induction of *vir*-genes by the addition of acetosyringone during the cocultivation period. The highest transformation frequency, 3% of the total number of explants cocultivated, was obtained with cotyledonary explants of cv. 'Pharo'. Although at a lower frequency (1.3%), we have also succeeded in the transformation of leaf explants. A loss of genetic material was detected in some plants, and results are in accordance with the directional model of T-DNA transfer. *In vitro* cultured shoots from transgenic populations carrying the *HAL1* gene were evaluated for salt tolerance on shoot growth medium containing 10 g l^{-1} NaCl. Although root and vegetative growth were reduced, transgenic *HAL1*-positive plants consistently showed a higher level of tolerance than control *HAL1*-negative plants.

Keywords: *Agrobacterium*-mediated transformation; *Cucumis melo*; transgenic plants; GUS expression; PCR analysis; salt tolerance

Introduction

Genetic engineering is a valuable biotechnological approach for breeding purposes. We are currently investigating the possibilities of applying such biotechnologies to genetically improve salt tolerance in certain crop species. Recently, significant advances have been made on the identification and isolation of genes involved in salt stress (see Serrano and Gaxiola, 1994). One of these genes, *HAL1* isolated from *Saccharomyces cerevisiae*, was obtained by a direct functional approach: yeast were transformed by a genomic library in a multicopy plasmid and transformants were screened for growth at high NaCl concentrations (Gaxiola *et al.*, 1992). *HAL1* encodes a watersoluble protein (32 kDa) that may modulate monovalent ion channels, by affecting the set point of intracellular potassium determined by the feedback regulation of the uptake system (Gaxiola *et al.*, 1992). This study indicated that genes homologous to the yeast *HAL1* could be present in higher plants, suggesting that this gene may be relevant to salt tolerance in higher plants.

Our first objective was to optimize a genetic transformation system that would permit the introduction of desirable genes into *Cucumis melo* cultivars. The availability of an efficient plant regeneration system from explant-derived calluses of melon (Moreno *et al.*, 1985; Moreno and Roig, 1990; García-Sogo *et al.*, 1991) and the selection of cultivars having a high regeneration capacity (Bordas *et al.*, 1991) enabled us to design the *Agrobacterium*-mediated gene transfer protocol described

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here. In order to optimize conditions for genetic transformation, the effects of several factors on transformation efficiency were investigated. The current study focused on the susceptibility of different cultivars, addition of acetosyringone and the selection procedure. In *C. melo* transformation systems have previously been reported, always using cotyledonary explants as starting plant material (Ben Tahar and De Both, 1988; Dong *et al.*, 1991; Yoshioka *et al.*, 1992; Fang and Grumet, 1993). We have transformed melon using this type of explant but we have also succeeded in the transformation of melon using leaf explants. It is useful to use alternative explant tissue (i.e. leaves) since regenerated plants could differ in ploidy levels as described for related species (Colijn-Hooymans *et al.*, 1988), and this could be of great relevance within the agronomic context.

Using *Agrobacterium* transformation we studied the expression of the *HAL1* gene in higher plants. Overexpression of the *HAL1* gene significantly improves yeast growth under salt stress and for this reason it seemed interesting to test whether its constitutive overexpression in transgenic melon plants could produce an increase in salt tolerance.

Materials and methods

Plant materials, culture media and in vitro culture conditions

Cotyledonary and leaf explants of cv. 'Pharo' (Sluis & Groot) and leaf explants of cv. 'Amarillo Canario' (Petoseed Ibérica S.A.) were used. Seeds were surface-sterilized in diluted commercial bleach (5% w/v sodium hypochlorite) with 0.1% (v/v) of 7X-O-matic (Flow Laboratories) for 30 min, and rinsed three times with sterile distilled water. After sterilization, the seeds were germinated on solid medium consisting of Murashige and Skoog's basal salts supplemented with 1% (w/v) sucrose, and 0.8% (w/v) agar (Industrial, Pronadisa).

Cotyledonary explants were taken from 1-week-old *C. melo* seedlings, by cutting each cotyledon into two segments. Apical shoots from these seedlings were cut above the cotyledons and rooted on BM3 solid medium (MS salts, 3% w/v sucrose, 100 mg l^{-1} myoinositol, 1 mg l^{-1} thiamine HCl). Leaf explants were prepared from 2-week-old *C. melo* plantlets by cutting 1 cm^2 segments of fully-expanded young leaves.

The callus induction medium consisted of BM3 basal medium supplemented with 1.5 mg l^{-1} indole acetic acid, 6 mg l^{-1} kinetin and 1 mg l^{-1} $CuSO_4$ $5H_2O$, which enhances shoot regeneration in melon (IKCu medium, García-Sogo *et al.*, 1991). In the regeneration medium the above growth regulators were replaced by 0.01 mg l^{-1} α-naphthalene acetic acid and 0.1 mg l^{-1} N^6-benzyladenine (NBCu medium). The regenerated shoots were rooted on the same basal medium (BM3) containing neither copper sulphate nor growth regulators.

Cultures were maintained in a tissue culture chamber at 24 °C ± 2 °C under a 16 h light/8 h dark photoperiod (cool white fluorescent light, 2000 lux). The pH of all media was adjusted with KOH to 5.7 before autoclaving at 115 °C for 30 min. Plant growth regulators were added to the media before autoclaving. Kanamycin, carbenicillin and acetosyringone were filter-sterilized and added to the autoclaved medium when needed. All media were solidified with 0.8% (w/v) Agar Industrial (Pronadisa).

Construction of binary plasmid pRS655 and Agrobacterium transformation

The complete coding region of the *HAL1* gene is available as a *Nco*I-*Pst*I fragment (Gaxiola *et al.*, 1992). The *Pst*I end was blunted with T4 DNA polymerase and the 0.9 kb *Nco*I-blunt fragment subcloned into plasmid pRT103 (Töpfer *et al.*, 1987) and digested with *Nco*I and *Sma*I to give plasmid pRS1055. This placed the *HAL1* coding region between the 35S promoter and the polyadenylation signal of the cauliflower mosaic virus (CaMV). The expression casette (promoter-*HAL1*-terminator) was obtained as a 1.6 kb *Hind*III fragment and subcloned into the *Hind*III site of binary plasmid pBI121 (Jefferson *et al.*, 1987) to give rise to pRS655 (14.1 kb). The orientation of the *HAL1* gene was determined by restriction digestion with *Bam*HI, since there is a *Bam*HI site in pRS1055 at the end of the *HAL1* reading frame. Another *Bam*HI site is present in the promoter of the *gus* gene of pBI121. A fragment of 2.1 kb was produced by *Bam*HI digestion of pRS655, demonstrating the structure of the T-DNA region depicted in Fig. 1. The three genes coding for *npt*II, *HAL1* and *gus* were transferred to the plant in the above order. Plasmid pRS655 was introduced into *Agrobacterium tumefaciens* strain LBA4404 (Hoekema *et al.*, 1983) by the method of Höfgen and Willmitzer (1988).

Transformation, selection and shoot regeneration

Transformation was performed essentially as described by Horsch and co-workers (1985) with some modifications.

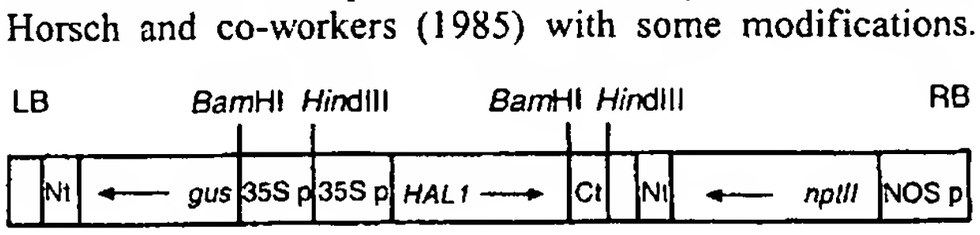

Fig. 1. Structure of the T-DNA region of binary plasmid pRS655. The 6.5 kb between the right (RB) and left (LB) borders defining the beginning and end of DNA transfer to the plant are shown. *NOS*p: nopaline synthase gene promoter; *nptII*: neomycin phosphotransferase gene conferring kanamycin resistance; Nt: nopaline synthase gene polyadenylation and transcription terminator site; 35Sp: 35S promoter of the CaMV virus; *HAL1*: *HAL1* coding region; Ct: polyadenylation and transcription termination site of CaMV virus; *gus*: β-glucuronidase coding region.

For infection, *Agrobacterium* cells were grown overnight on a rotary shaker (250 rpm) at 24–26 °C in liquid Luria-Bertrani broth (Bacto-tryptone 10 g l^{-1}, Bacto-yeast extract 5 g l^{-1}, NaCl 10 g l^{-1}), supplemented with 50 mg l^{-1} kanamycin . Explants were inoculated with an overnight culture of *Agrobacterium* diluted to OD_{600} 0.8 (10^7–10^8 bacteria ml^{-1}) for 10 min, dried on blotting paper to remove excess bacteria and cocultivated for 3 days at 28 °C in the dark on callus induction IKCu medium. Bacteria were then removed by washing with liquid MS medium containing 500 mg l^{-1} carbenicillin, explants were transferred onto selective callus induction IKCu medium containing 100 mg l^{-1} kanamycin and 300 mg l^{-1} carbenicillin. After 4 weeks of incubation, green kanamycin-resistant calluses were transferred to the regeneration NBCu medium containing 100 mg l^{-1} kanamycin and 300 mg l^{-1} carbenicillin. Regeneration of shoots was obtained from these green resistant calluses through several subcultures under the same selective pressure. These shoots were transferred to rooting BM3 medium without antibiotics. Kanamycin has a negative effect on root formation and was omitted during this step. Putative transgenic plants were then tested for transformation using GUS activity, PCR analysis and Southern hybridization. Rooted transformants were either maintained in this medium or transferred to soil and grown to maturity under greenhouse conditions. From here on, we designated this protocol as standard, and all subsequent experiments were conducted in this way, unless otherwise noted.

To evaluate the susceptibility of different genotypes to *A. tumefaciens* strain LBA4404, leaf explants of both cultivars 'Pharo' and 'Amarillo Canario' were used and transformed under the conditions described above. Transfer time to selective medium was evaluated in a second experiment using cotyledonary explants of cv. 'Pharo'. In this case, modifications included two different selection procedures: selection starting either immediately after infection, by adding 50 mg l^{-1} kanamycin to the cocultivation medium (continuous selection), or after cocultivation for 3 days without selective pressure (standard selection), followed by transfer to selective IKCu medium with 100 mg l^{-1} kanamycin. In the third experiment, leaf explants of cv. 'Amarillo Canario' were used; acetosyringone (200 μM) was added to the cocultivation medium, and the effect of the presence/absence of acetosyringone (200 μM) in the overnight bacterial culture was tested. Sets of negative controls were always included, consisting of explants immersed in bacteria-free LB medium, under the same conditions as each experiment.

Assay for β-glucuronidase activity in the transformed plants

The expression of the β-glucuronidase (*gus*) gene was assayed in all putative transformed plants regenerated on selective media. Fluorimetric determination of GUS activity in the plant tissues was performed according to Jefferson and co-workers (1987). Leaf tissue from negative controls and putative transformed plants was homogenized with extraction buffer (100 mg tissue ml^{-1}) and the fluorimetric reaction was carried out in 80 μl reaction buffer containing 1 mM 4-methylumbelliferyl-β-D-glucuronide (Sigma, M-9130) as substrate. The fluorescence emitted by the formation of 4-methylumbelliferone was measured after incubation for 12 h. Several shoots from the same clone were tested, and assays were repeated at least twice in subsequent transfers.

DNA preparation and polymerase chain reaction (PCR) analysis

Polymerase chain reaction was used to detect specific DNA sequences of the three genes transferred: *nptII*, *HAL1* and *gus*. DNA from 0.5–1 g of plant leaves was prepared by the simplified CTAB (cetyl trimethyl ammonium bromide) method of Doyle and Doyle (1990). Yield was 30–60 μg. Polymerase chain reaction detection of β-glucuronidase (*gus*), neomycin phosphotransferase (*nptII*) and *HAL1* genes was performed with standard methods (Taylor, 1991). Briefly, 0.2 μg of DNA was incubated in a final volume of 50 μl with 0.25 μg of forward and reverse primers, 0.2 mM each of dATP, (dGTP, dCTP and dTTP and two units of thermostable DNA polymerase from *Thermus brockianus* (Dynazyme, Finnzymes OY, Finland). Reaction cycles (25 cycles) consisted of 30 s at 95 °C for denaturation, 30 s at 60 °C for annealing and 60 s at 70 °C for extension. The *HAL1* primers have been described (Gaxiola *et al.*, 1992) and they amplify a fragment of 888 bp. The forward and reverse primers for *gus* gene were 5′-ATCAGGAAGTGATGGAGCATCA and 5′-GGTGATCGGACGCGTCGGGTCG, respectively, amplifying a fragment of 1021 bp from position 601 to 1622 of the sequence (Jefferson *et al.*, 1986). The forward and reverse primers for the *nptII* gene were 5′-AAGATGGATTGCACGCAGGTTC and 5′-GAAGAACTCGTCAAGAAGGCGA, respectively, amplifying a fragment of 781 bp from position 161 to 942 of the sequence (Beck *et al.*, 1982).

Northern and Southern analysis

Total RNA was extracted from leaf tissue using the Ecker and Davis method (1987), electrophoresed on 1% agarose gels with 2% formaldehyde, transferred to nylon membranes (Kroczek and Siebert, 1990) and hybridized with the *Nco* I-*Pst* I fragment containing the *HAL1* gene labelled by the Feinberg and Vogelstein method (1983).

For Southern analysis, total DNA was extracted as described (Doyle and Doyle, 1990), digested with *Xba* I, separated by electrophoresis in agarose gels, transferred to nylon membranes and hybridized with the *HAL1* probe as described above.

Evaluation of salt tolerance

Cucumis melo L. cv. 'Pharo' was used. Apical shoots, 2 cm long, were excised from 2-week-old *in vitro* plants and transferred to BM3 medium containing 10 g l^{-1} NaCl. After 16 days of culture *HAL1*-positive and non-transgenic plants were evaluated for root and shoot growth: percentage of shoots forming primary roots, the number of primary roots and their length, plant height, vegetative fresh weight, elongation of the first internode (from the cotyledonary node up to the first leaf node) and leaf number. Two sorts of *HAL1*-positive plants and two sets of controls were tested: (1) leaf-derived *HAL1*-positive plants (L_*HAL1*-positive, 182 replicates from 25 individual plants), (2) cotyledon-derived *HAL1*-positive plants (C_*HAL1*-positive, 52 replicates from 12 individual plants), (3) escape *HAL1*-negative plants from the transformation experiments (S_*HAL1*-negative, 47 replicates from 11 individual plants), (4) cotyledon-derived untransformed plants obtained through the same regeneration protocol (C_*HAL1*-negative, 30 replicates from 30 individual plants).

Data analysis

The transformation frequencies were calculated as the percentage of calluses forming transgenic shoots over the total number of explants treated. Data were analysed by analysis of variance (ANOVA). The Least Significative Differences (LSD) test was used to compare the performance under salt stress of the different populations tested. Results expressed as a percentage of explants form a binomial distribution and were transformed $(\arcsin(P)^{0.5})$ before analysis; data collected as counts were transformed using $(x+0.5)^{0.5}$ before analysis, to correct deviations from the basic assumptions of ANOVA.

Results

Regeneration of transgenic plants

An initial experiment was carried out to investigate the susceptibility of leaf explants of cvs 'Pharo' and 'Amarillo Canario' to *A. tumefaciens* strain LBA4404. Results of the different responses from both cultivars are summarized in Table 1. The inoculated explants developed green organogenic calluses on selective IKCu medium containing 100 mg l^{-1} kanamycin and 300 mg l^{-1} carbenicillin. Several green calluses of cv. 'Pharo' regenerated shoots when transferred to NBCu medium under the same selection pressure, at a frequency of 0.6% over the total number of explants treated. Although previous experiments had shown that regeneration from leaf explants of cv. 'Amarillo Canario' could be readily obtained (Bordas *et al.*, 1991), inoculated explants did not regenerate when cultured on selection media containing kanamycin. Leaf explants of cv. 'Pharo' formed transgenic shoots, but transformation frequency was low (0.4%).

To test different selection procedures following infection, inoculated and control cotyledonary explants of cv. 'Pharo' were placed on cocultivation IKCu medium containing 50 mg l^{-1} kanamycin for 3 days (continuous selection) and in the same medium without the antibiotic (standard selection). After this period, all explants were transferred to the selective IKCu medium containing 100 mg l^{-1} kanamycin. The effects of continuous kanamycin selection compared to standard selection are summarized in Table 1. The response of cotyledonary explants of cv. 'Pharo' differed between the selection procedures. Standard selection resulted in better callus formation than continuous selection, and subsequently yielded about twofold higher shoot regeneration. Each selection procedure yielded transformed shoots, but an average frequency of 1% independent transgenic plants

Table 1. Transformation frequencies of different melon cultivars and transformation protocols

Cultivar	*Type of explant*	*Method*[a]	*Total no. explants tested*	*Explants with green calluses (%)*[b]	*Explants with shoots (%)*[c]	*Explants with transformed plants (%)*[d]
Pharo	Leaf	1	177	0	0	0
Pharo	Leaf	2	486	8 (1.7)	3 (0.6)	2 (0.4)
Pharo	Cotyledon	1	200	0	0	0
Pharo	Cotyledon	2	303	25 (8.3)	13 (4.3)	10 (3)
Pharo	Cotyledon	3	296	10 (3.4)	7 (2.4)	3 (1)
Amarillo canario	Leaf	1	252	0	0	0
Amarillo canario	Leaf	2	238	1 (0.4)	0	0
Amarillo canario	Leaf	4	151	11 (7.3)	2 (1.3)	1 (0.7)
Amarillo canario	Leaf	5	150	8 (5.3)	4 (2.7)	2 (1.3)

[a]Method 1: transformation protocol without *Agrobacterium*; method 2: standard transformation protocol; method 3: kanamycin (50 mg l^{-1}) present during cocultivation; method 4: acetosyringone (200 μM) present during cocultivation; method 5: acetosyringone (200 μM) present during bacterial growth and during cocultivation.
[b]Data scored after 4 weeks in selective IKCu medium containing 100 mg l^{-1} kanamycin and 300 mg l^{-1} carbenicillin.
[c]Regeneration frequency; data compiled after subculturing from first to sixth subculture on selective NBCu medium containing 100 mg l^{-1} kanamycin and 300 mg l^{-1} carbenicillin.
[d]Transgenic plants detected by GUS assays and PCR analysis.

obtained under continuous selection was significantly lower than that of 3% under standard selection.

In the first experiment we were unable to regenerate plants from inoculated leaf explants of cv. 'Amarillo Canario' when selected on kanamycin exposed to the standard treatment. As shown in Table 1, shoot regeneration in medium containing kanamycin (100 mg l^{-1}) was achieved when cocultivation media were supplemented with 200 µM acetosyringone. Although transformation frequency was slightly higher (1.3% vs 0.7%), there was no significant improvement with the addition of acetosyringone to the bacterial growth medium.

Controls were carried out simultaneously in all experiments; uninoculated explants never regenerated using the same concentration of kanamycin (100 mg l^{-1}).

GUS activity in the transformed plants

Preliminary and simultaneous sets of control experiments demonstrated that GUS expression was never detectable in untransformed control plants, indicating that there was no intrinsic GUS activity under our experimental conditions. As the chimaeric construct used in our transformations is also expressed by *Agrobacterium* cells, prior to performing GUS assays, plants were transferred to antibiotic-free media to ensure that the material was *Agrobacterium* free. Under these conditions, without antibiotics, no bacterial growth was observed in any of the subsequent culture transfers. GUS activity assays were performed with plants grown on antibiotic-free media. Therefore, the possibility of enzymatic GUS activity due to bacterial contamination persisting in the tissues can be discounted.

In order to evaluate whether the GUS assay is a reliable test of the transgenic status, the correlation between GUS expression and the presence of the gene was analysed. We found a correlation $r = 0.95$; all of the transgenic plants showing GUS activity by fluorimetric assay were PCR-*gus*-positive. The absence of GUS activity in fluorimetric assays also correlated very well with the absence of the gene. The only exception was a single plant that failed to exhibit GUS expression although the marker gene was detected by PCR analysis (Table 2).

Co-integration of the transferred genes

Figure 2 illustrates the predictable fragments following PCR amplifications with each pair of primers (*nptII*, *HAL1* and *gus*). Since we observed that not all transgenic plants contained the complete T-DNA insert, fragmentation events and losses of genetic material were also quantified. Frequencies of integration for each of the three genes in the different transformation experiments are shown in Table 2. The *nptII* gene was present in all transgenic plants, the *gus* gene was lost at a higher frequency than *HAL1* gene and in the case where *HAL1* gene was missing the *gus* gene also proved to be so.

Efficiency of the selection procedure

The relationship between phenotypic resistance to kanamycin and presence of the *nptII* gene was analysed to

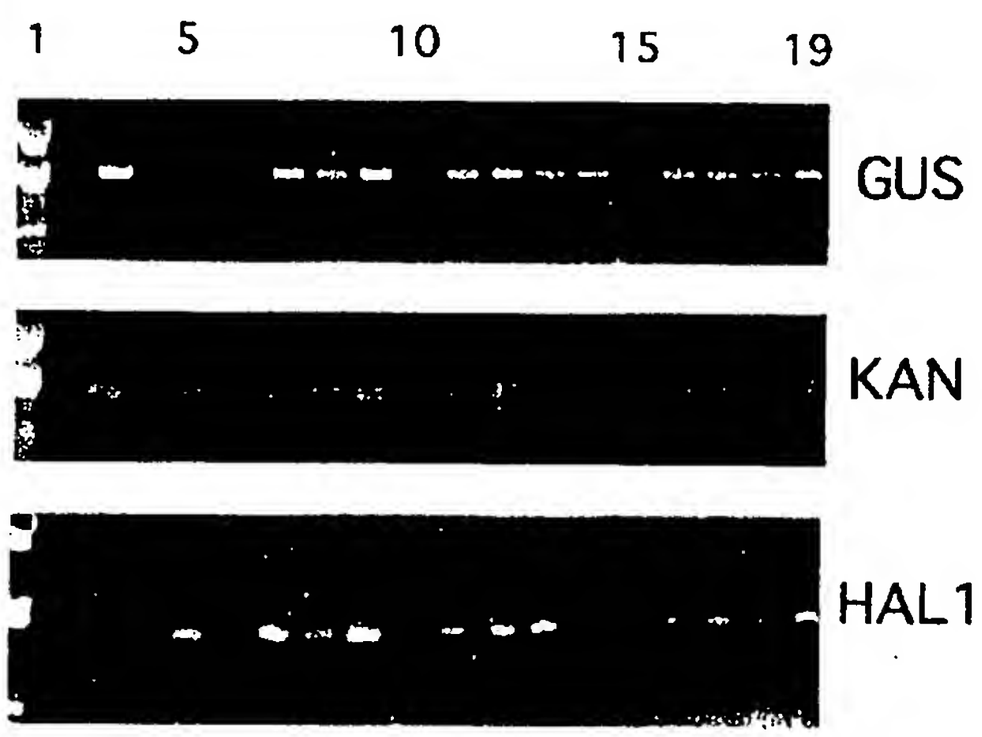

Fig. 2. Electrophoretic analysis of the PCR products of putative transgenic plants. GUS, KAN and HAL1 indicates PCR reactions with the primers of β-glucuronidase, neomycin phosphotransferase and *HAL1*, respectively. Aliquots of 5 µl from the samples described below were analysed. (1) size standards of 1.9 (top), 1.1, 0.9 and 0.4 kb. (2) negative control without DNA. (3) control with pBI121 plasmid. (4) negative control of DNA from non-transgenic plant. (5) partial transgenic plant. (6, 10, 15) escape plants. (7, 8, 9, 11, 12, 13, 14, 16, 17, 18, 19) transgenic plants.

Table 2. Frequencies of co-integration of *nptII*, *HAL1* and *gus* genes in transgenic plants

	PCR analysis				
Class	nptII	HAL1	gus	*GUS assay*	*Total number (%)*
Full transgenic	+	+	+	+	24 (82.7)
	+	+	+	–	1 (3.5)
Partial	+	+	–	–	3 (10.3)
Partial	+	–	–	–	1 (3.5)

evaluate the efficiency of the conditions and the selective schemes tested. When comparing the frequency of transgenic plants between both selection schemes, noticeable differences were found. As seen in Table 3, when imposing standard selection the frequencies of PCR-*nptII*-positive calluses over the total number of kanamycin-resistant calluses were 67% and 75% when leaf and cotyledonary explants of cv. 'Pharo' were transformed, respectively. The frequency of transgenic PCR-*nptII*-positive shoots under continuous selection was lower (50%). Within the PCR-*nptII*-positive calluses we distinguished chimaeric calluses, which are those occasionally producing PCR-*nptII*-negative plants. These chimaeric calluses were only observed under standard conditions.

Sexual transmission of the transgene

The primary transformants were acclimatised under greenhouse conditions, self-pollinated and their progeny was analysed for segregation of the transgenes and phenotypes (in preparation). The demonstration of sexual transmission of the *HAL1* gene within a transgenic line is shown in Fig. 3. A 2.1 kb band recognized by the *HAL1* probe is present in a primary transformant and its progeny but absent in a untransformed control plant.

Northern analysis

(The level of expression of the *HAL1* gene in transgenic melon plants was investigated by northern analysis (Fig. 4). Most transgenic plants (eight out of 12 analysed) exhibited significant levels of *HAL1* mRNA which coud be detected by hybridization of total RNA. As expected (Finnegan and McElroy, 1994), the level of expression is variable, undetectable in some plants (lanes 2 and 9) and very high in others (lanes 4–6). One plant (lane 3) exhibited an unusually large hybridizing band in addition to the expected at about 1 kb. In plants with high expression (lanes 5–7) the smearing below the major band could be due to either truncated or degraded messages. No evidence for degradation of the RNA was observed in ethidium-bromide stained RNA gels.

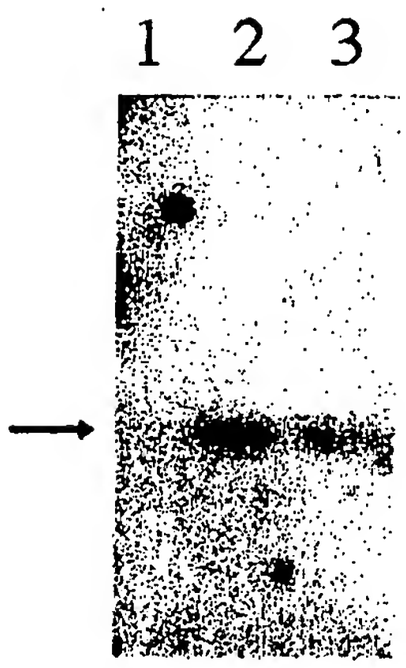

Fig. 3. Southern analysis of the transmission of *HAL1* during sexual reproduction. Two µg DNA from an untransformed control plant (lane 1), a primary transformant (lane 2) and one plant from the second generation progeny of the same self-fertilized primary transformant (lane 3), were probed by Southern analysis as described in Materials and Methods. Arrow on the left indicates the hybridizing band of 2.1 kb recognized by the *HAL1* probe.

Salt tolerance in transgenic plants carrying the HAL1 gene

Under stress conditions (10 g l^{-1} NaCl during 16 days) transgenic and control populations differed in their behaviour. The frequency and intensity of root formation were higher in *HAL1*-positive populations compared to control populations. Specific mean comparisons showed significant differences ($P < 0.05$) between L_*HAL1*-positive plants and both control *HAL1*-negative populations for all the variables related to rooting. Also the number of primary roots differed significantly between C_*HAL1*-positive plants and both control populations, and in all cases significant differences were found between C_*HAL1*-positive and C_*HAL1*-negative plants (Fig. 5A–C). Results related to vegetative growth were not as clear as those observed for root growth. No differences between populations in vegetative fresh weight and number of leaves were detected. However, plant height and the elongation of the first internode in *HAL1*-positive plants

Table 3. Frequencies of explants regenerating PCR-*nptII*-positive calluses from transformation experiments of *C. melo* cv. 'Pharo'

			nptII-*positive calluses*			nptII-*negative calluses*
Type of explant	*Selection procedure*[a]	*No. of calluses tested*	*Non-chimaeric*	*Chimaeric*	*Selection efficiency (%)*	*Frequency of escapes (%)*
Leaf	Standard	3	1	1	66.7	1 (33.3)
Cotyledon	Standard	12	6	3	75	3 (25)
Cotyledon	Continuous	6	3	0	50	3 (50)

[a]Standard: explants cocultivated with *Agrobacterium* on IKCu medium without kanamycin for 3 days and subsequently transferred to selective IKCu medium with 100 mg l^{-1} kanamycin; continuous: explants cocultivated with *Agrobacterium* on IKCu with 50 mg l^{-1} kanamycin for 3 days and subsequently transferred to selective IKCu medium with 100 mg l^{-1} kanamycin.

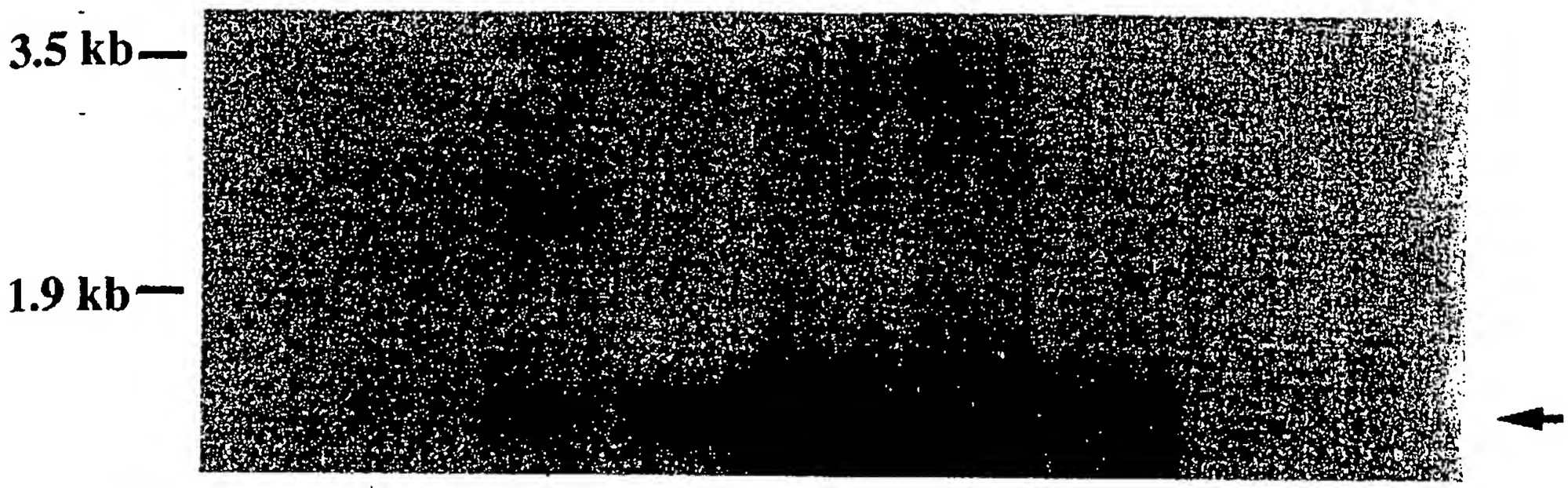

Fig. 4. Northern analysis of *HAL1* expression in transgenic melon plants. Twenty μg of total RNA was applied per lane. (1) control (non-transgenic) plant; (2–9) transgenic plants. The position of ribosomal RNAs of 3.5 and 1.9 kb is indicated on the left. The arrow on the right marks the position of the *HAL1* mRNA of about 1 kb. In lanes 5–8 some smearing due to truncated or degraded messages is apparent below the major band at about 1 kb. In lane 3, in addition to the normal *HAL1* mRNA, a second larger message is observed (indicated by an asterisk).

consistently reached higher values than control plants (data not shown). Although the differences found in plant height were not significant, again the elongation of the first internode was significantly greater in *HAL1*-positive populations.

Discussion

The results presented here show that cultivars of *C. melo* differ in their response to the same standard conditions of transformation. Leaf explants of cv. 'Amarillo Canario' resulted in infrequent callus formation and no shoot regeneration under conditions which resulted in transformation for leaf explants of cv. 'Pharo', even though without kanamycin leaf explants of cv. 'Amarillo Canario' showed a higher morphogenetic response than leaf explants of cv. 'Pharo'. This may be attributed to a difference in susceptibility of the two genotypes to a specific strain of *A. tumefaciens*, or alternatively, to a difference in optimal transformation conditions, such as antibiotic sensitivity. Differences in susceptibility of species, lines and genotypes within the same species are common and have been reported previously (Knauf *et al.*, 1982; Smarrelli *et al.*, 1986).

A major problem in many transformation methods is the occurrence of a high number of escape shoots under kanamycin selection (Dong *et al.*, 1991). In our experimental designs two selection schemes were proposed: a standard protocol, with a 3-day delay between the infection and the application of kanamycin selection, and a protocol using continuous selection. It seemed logical to think that continuous selection would facilitate the stabilization of the binary plasmid which, in addition to T-DNA, contains a bacterial gene for kanamycin resistance. Indeed, it has been demonstrated that these vectors are easily lost from *A. tumefaciens* in the absence of selection. However, the standard selection scheme produced the highest number of regenerants. Continuous selection caused a reduction of regenerants without affecting the number of calluses that escaped the kanamycin selection. This suggests that a certain period in the absence of selection (i.e. without kanamycin) is needed to allow T-DNA transfer, integration, transcription and sufficient NPTII enzyme production leading to the expression of a kanamycin-resistant phenotype. Loss of the bacterial plasmid due to the absence of selection did not apparently influence the transformation efficiency. We concluded therefore that the standard protocol was beneficial in ensuring a high transformation frequency.

Several studies have demonstrated that acetosyringone increases transformation efficiency in various species (Godwin *et al.*, 1991; Sarmento *et al.*, 1992; Yoshioka *et al.*, 1992). As described for muskmelon by Yoshioka and co-workers (1992), we also observed a positive effect of acetosyringone on transformation efficiency during co-cultivation period. In fact, the acquisition of transgenic plants of cv. 'Amarillo Canario' was only achieved after induction of the *vir*-genes by addition of acetosyringone (200 μM) to the cocultivation medium. However, the use of acetosyringone during bacterial growth (in addition to the cocultivation period) did not have any apparent influence (did not differ significantly at $P < 0.05$).

We have noted a correlation between GUS expression and molecular analysis which confirms that GUS activity

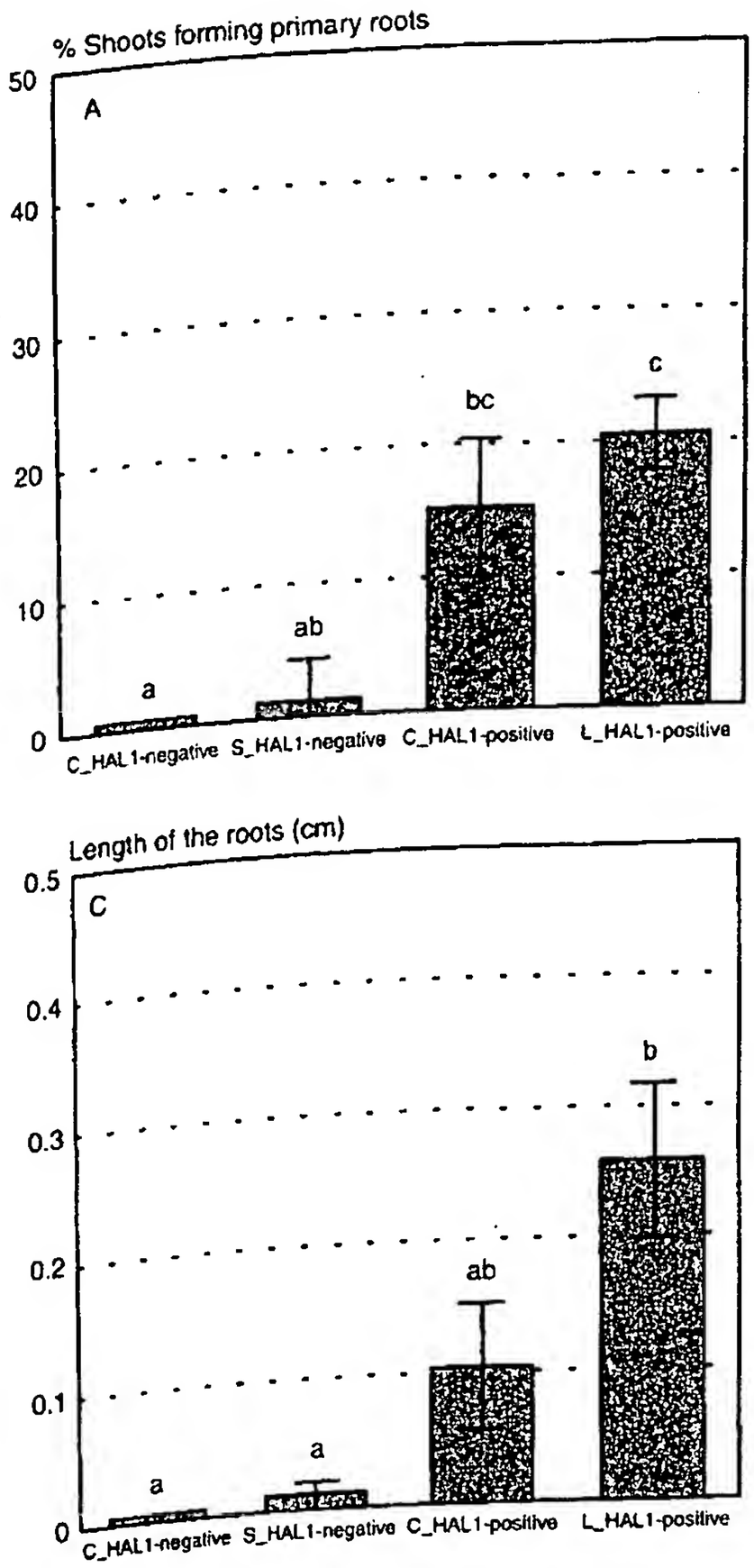

Fig. 5. Growth responses to NaCl of the four different populations tested: C_*HAL1*-negative, S_*HAL1*-negative, C_*HAL1*-positive, L_*HAL1*-positive. (A) percentage of shoots forming primary roots, (B) number of primary roots, (C) length of the roots. Responses were evaluated by culturing shoots on BM3 medium supplemented with $10\,g\,l^{-1}$ NaCl after 16 days growth. Means with the same letter are not significantly different at $P < 0.05$ (LSD: Least Significant Difference test). Error bars are SE.

in the cells of transgenic melon plants is indeed due to the integration and transcription of the foreign gene. We concluded that the CaMV 35S promoter is a good choice for the expression of foreign genes in melon.

The data of co-integration of the transferred genes in our analysis are in accordance with the directional model of T-DNA transfer, from the right border to the left border (Wang *et al.*, 1984). The predominance of *nptII* integration is not surprising, since selection is based on this gene, and it is located close to the right border. The most distant *gus* gene, from the right border, is lost at a higher frequency. This suggests that losses of genetic material are mostly due to incomplete T-DNA transfer during integration into the plant genome. Our results agree with those obtained by Chee and Slightom (1991) in cucumber, who also reported the generation of kanamycin-resistant transgenic plants which do not possess the *gus* gene, according to directional transfer.

In order to determine the appropriate test and concentration of NaCl, the sensitivity of shoots to NaCl was studied prior to evaluating salt tolerance (data not shown). On the basis of this previous study, we were able to define several parameters and chose an appropriate level of stress (i.e. $10\,g\,l^{-1}$ NaCl during 16 days) to effectively discriminate salt tolerant plants. Under these conditions a higher level of tolerance to NaCl in transgenic *HAL1*-positive populations compared to control populations was demonstrated. The variability found in response to salt stress could be explained by different mechanisms. One explanation might be that the varia-

bility for salt tolerance was already present in the original population, as reported for other species (Akbar *et al.*, 1972; Maas and Hoffman, 1977; Shannon, 1978). Another explanation might be the generation of variability as a result of somaclonal variation phenomena, either genetic or epigenetic. However, none of these hypotheses explain the consistent differences between the *HAL1*-positive and *HAL1*-negative populations since a similar degree of variation and the same probability of somaclonal variation events would be expected. The evidence from our research strongly suggests that the higher level of tolerance observed in *HAL1*-positive plants arises from *HAL1* gene expression.

Expression of *HAL1* gene was also studied by determining specific mRNA in transgenic plants by Northern blot. The results from these experiments confirmed the presence of significant amounts of RNA transcript of the expected size, thus providing further evidence of the expression of the *HAL1* gene in higher plants.

Other attempts to confer tolerance against osmotic stresses by genetic engineering have been reported. Tarczynski and co-workers (1993) engineered transgenic tobacco plants that synthesize and accumulate mannitol by the introduction of a bacterial gene (mannitol 1-phosphate dehydrogenase) and plants containing mannitol had an increased ability to tolerate salt stress. Recently, Pilon-Smits and co-workers (1995) have reported enhanced resistance to drought stress in transgenic tobacco plants that accumulate bacterial fructans; and Kishor and co-workers (1995) have demonstrated that the overproduction of proline results in increased tolerance to osmotic stress (drought and saltinity) in transgenic tobacco plants. Here we demonstrate that it is possible to increase salt tolerance of *in vitro* cultured melon by introducing the foreign gene *HAL1* isolated from yeast. The nature of these changes at the molecular level still needs elucidating. In yeast *HAL1* functions by decreasing the Na^+/K^+ ratio (Gaxiola *et al.*, 1992). The operation of a similar mechanism in transgenic melon is being studied. Additional study is needed to evaluate whether the selected transformants exhibit relevant levels of salt tolerance *in vivo*, under greenhouse or field conditions.

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References

Akbar, M., Yabuno, T. and Nakao, S. (1972) Breeding for saline resistant varieties of rice. I. Variability for salt tolerance among some rice varieties. *Jap. J. Breed.* **22**, 272–84.

Beck, E., Ludwig, G., Auerswald, E.A., Reiss, B. and Schaller, H. (1982) Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. *Gene* **19**, 327–36.

Ben Tahar, S. and De Both, M.T.J. (1988) Introduction of foreign genes into melon (*Cucumis melo* L.) using *Agrobacterium tumefaciens*. In: Risser, G. and Pitrat, M., eds, *Cucurbitaceae 88, Proc. Eucarpia Cucurbit Genetics and Breeding*, pp. 209–16, Avignon-Montfavet, France.

Bordas, M., Moreno, V. and Roig, L.A. (1991) Organogenic amd embryogenic potential of several commercial lines of *Cucumis melo* L. *Cucurbit Gen. Coop. Rep.* **14**, 71–3.

Chee, P.P. and Slightom, J.L. (1991) Transfer and expression of cucumber mosaic virus coat protein gene in the genome of *Cucumis sativus*. *J. Amer. Soc. Hort. Sci.* **116**, 1098–102.

Colijn-Hooymans, C.M., Bower, R., Orczy, W. and Dons, J.J.M. (1988) Plant regeneration from cucumber (*Cucumis sativus*) protoplasts. *Pl. Cell Rep.* **7**, 626–7.

Dong, J-Z., Yang, M-Z., Jia, S-R. and Chua, N-H. (1991) Transformation of melon (*Cucumis melo* L.) and expression from the cauliflower mosaic virus 35S promoter in transgenic plants. *Bio/Technol.* **9**, 858–63.

Doyle, J.J. and Doyle, J.L. (1990) Isolation of plant DNA from fresh tissue. *Focus* **12**, 13–15.

Ecker, J.R. and Davis, R.W. (1987) Plant defense genes are regulated by ethylene. *Proc. Natl. Acad. Sci. USA* **84**, 5202–6.

Fang, G. and Grumet, R. (1993) Genetic engineerring of potyvirus resistance using constructs derived from the zucchini yellow mosaic virus coat protein gene. *Mol. Plant-Microbe Interact.* **6**, 358–67.

Feinberg, A.P. and Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6–13.

Finnegan, J. and McElroy, D. (1994) Transgene inactivation: plant fight back. *Bio/Technol.* **12**, 883–8.

García-Sogo, B., Roig, L.A. and Moreno, V. (1991) Enhancement of morphogenetic response in cotyledon-derived explants of *Cucumis melo* induced by copper ion. *Acta Hort.* **289**, 229–30.

Gaxiola, R., de Larrinoa, I.F., Villalba, J.M., and Serrano, R. (1992) A novel and conserved salt-induced protein is an important determinant of salt tolerance in yeast. *EMBO J.* **11**, 3157–64.

Godwin, I., Todd, G., Ford-Lloyd, B. and Newbury, H.J. (1991) The effects of acetosyringone and pH on *Agrobacterium*-mediated transformation vary according to plant species. *Pl. Cell Rep.* **9**, 671–5.

Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J. and Schilperoort, R.A. (1983) A binary plant vector strategy based on separation of vir- and T-region of the *Agrobacterium tumefaciens* Ti-

plasmid. *Nature* **303**, 179–80.

Höfgen, R. and Willmitzer, L. (1988) Storage of competent cells for *Agrobacterium* transformation. *Nucl. Acids Res.* **16**, 9877.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rodgers, S.G. and Fraley, R.T. (1985) A simple and general method for transferring genes into plants. *Science* **227**, 1229–31.

Jefferson, R.A., Burgess, S.M. and Hirsch, D. (1986) β-glucuronidase from *Escherichia coli* as a gene fusion marker. *Proc. Natl Acad. Sci. USA* **83**, 8447–51.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: β-glucuronidase an versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901–7.

Kishor, P.B.K., Hong, Z., Miao, G.-H., Hu, C.-A.A. and Verma, D.P.S. (1995) Overexpression of Δ^1-Pyrroline-5-Carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiol.* **108**, 1387–94.

Knauf, V., Panagopoulus, C. and Nester, E. (1982) Genetic factors controlling the host range of *Agrobacterium tumefaciens*. *Phytopathol.* **72**, 1545–9.

Kroczek, R.A. and Siebert, E. (1990) Optimization of Northern analysis by vacuum-blotting, RNA transfer visualization and ultraviolet fixation. *Anal. Biochem.* **184**, 90–5.

Maas, E.V. and Hoffman, G.J. (1977). Crop salt tolerance – current assessment. *J. Irrigation and Drainage Div. ASCE* **103**, 115–34.

Moreno, V. and Roig, L.A. (1990) Somaclonal variation in Cucurbits. In Bajaj, Y.P.S. (ed) *Biotechnology in Agriculture and Forestry: Somaclonal Variation in Crop Improvement I*, Vol. 11. pp. 435–64. Berlin, Heidelberg: Springer-Verlag.

Moreno, V., Garcia-Sogo, B., Granell, I., Garcia-Sogo, B. and Roig, L.A. (1985) Plant regeneration from calli of melon (*Cucumis melo* L. cv. 'Amarillo Oro'). *Pl. Cell Tiss. Organ Culture* **5**, 139–46.

Pilon-Smits, E.A.H., Ebskamp, M.J.M., Paul, M.J., Jeuken, M.J.W., Weisbeek, P.J. and (Smeekens, S.C.M. (1995) Improved performance of transgenic fructan-accumulating tobacco under drought stress. *Plant Physiol.* **107**, 125–30.

Sarmento, G.G., Alpert, K., Tang, F.A. and Punja, Z.K. (1992) Factors influencing *Agrobacterium tumefaciens* mediated transformation and expression of kanamycin resistance in pickling cucumber. *Pl. Cell Tiss. Organ Culture* **31**, 185–93.

Serrano, R. and Gaxiola, R. (1994) Microbial models and salt stress tolerance in plants. *Crit. Rev. Plant Sci.* **13**, 121–38.

Shannon, M.C. (1978). Testing salt tolerance variability among tall wheatgrass populations. *Agron. J.* **70**, 719–22.

Smarrelli, J.R., Watters, M.T. and Diba, L.H. (1986) Response of various cucurbits to infection by plasmid-harboring strains of *Agrobacterium*. *Pl. Physiol.* **82**, 622–4.

Tarczynski, M.C., Jensen, R.G. and Bohnert, H.J. (1993) Stress protection of transgenic tobacco by production of the osmolyte mannitol. *Science* **259**, 508–510.

Taylor, G.R. (1991). Polymerase chain reaction: basic principles and automation. In McPherson, M.J., Quirke, P. and Taylor, G.R. (eds) *PCR. A Practical Approach*. pp. 1–14. Oxford: IRL Press.

Töpfer, R., Matzeit, V., Gronenborn, B., Schell, J. and Steinbiss, H.-H. (1987) A set of plant expression vectors for transcriptional and translational fusions. *Nucl. Acids Res.* **15**, 5890.

Wang, K., Herrera-Estrella, L., van Montagu, M. and Zambryski, P. (1984) Right 25 bp terminus sequence of the nopaline T-DNA is essential for and determines direction of DNA transfer from *Agrobacterium* to the plant genome. *Cell* **38**, 455–62.

Yoshioka, K., Hanada, K., Nakazaki, Y., Minobe, Y., Yakuwa, T. and Oosawa, K. (1992) Successful transfer of the cucumber mosaic virus coat protein gene to *Cucumis melo* L. *Jap. J. Breed.* **43**, 629–34.

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Agrobacterium tumefaciens-mediated transformation of *Brassica napus* winter cultivars

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An efficient protocol for *Agrobacterium tumefaciens*-mediated transformation of six commercial *Brassica napus* winter cultivars is described. Two *B. napus* spring cultivars were analysed for comparison. Five strains of *A. tumefaciens* with different combinations of nopaline and octopine chromosomal backgrounds and virulence plasmids were used for cocultivation. Selection of putative regenerated transgenic plants was performed on kanamycin- or hygromycin-containing media. The scores of transgenic plants were calculated on the basis of GUS (β-glucuronidase) activity, detected by the histochemical X-Gluc test. Target tissue derived from the cut surface of cotyledon petioles resulted in successful transformation with all the winter cultivars tested. Target tissue from hypocotyl segments resulted in a successful transformation with only one winter cultivar. The transformation rates for *B. napus* winter cultivars in this study were higher than in previous reports. Southern blot analysis revealed that integration of marker genes occurred in single and in multiple copies and at multiple loci in the genome. The transgenic plants all grew normally and developed fertile flowers after a vernalization period. After self-pollination, Southern blot analysis of selected GUS active F_1 plants revealed that introduced marker genes were stably inherited to the next generation. These data demonstrate that morphologically normal, fertile transgenic plants of *B. napus* winter cultivars can be achieved with both nopaline- and octopine-derived *A. tumefaciens* strains. This protocol should have a broad application in improvement of *Brassica napus* winter cultivars by introduction of foreign genes.

Keywords: *Agrobacterium tumefaciens*; *Brassica napus*; winter cultivar; transformation; GUS; hygromycin; kanamycin

Introduction

Oilseed rape (*Brassica napus*) is the most important crop for production of vegetable oils in Northern Europe, Canada and China (Downey and Röbbelen, 1989). Furthermore, rapeseed is a plant species amenable to many tissue- and cell-culture techniques (Sjödin, 1992). Several procedures for genetic transformation of *B. napus* have been reported, such as microinjection (Spangenberg *et al.*, 1986), direct DNA uptake (Rasmussen and Rasmussen, 1993), microprojectile bombardment (Chen and Beversdorf, 1994) as well as *Agrobacterium*-mediated DNA transfer (Fry *et al.*, 1987; Pua *et al.*, 1987; De Block *et al.*, 1989; Moloney *et al.*, 1989; Boulter *et al.*, 1990; Damgaard and Rasmussen, 1991; Schröder *et al.*, 1994; Stefanov *et al.*, 1994). *Agrobacterium tumefaciens*-mediated transformation has proven to be highly efficient in many *Brassica* species. Using genetic transformation, new traits such as modifications of the oil composition (Knutzon *et al.*, 1992), herbicide tolerance (De Block *et al.*, 1989; De Block and Debrouwer, 1991) and altered protein composition (Altenbach *et al.*, 1992) have been introduced into *B. napus*.

It is well established that several experimental factors may affect the transformation process and the regeneration of transgenic plants. Amongst them, the genotype of the plant, the *A. tumefaciens* strain (chromosomal background, virulence genes), the inoculation method, the physiological condition of the target cells and the selection procedure of the putative transgenic shoots have been found to be crucial. For *A. tumefaciens*-mediated transformation of *B. napus* it has been reported

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that vectors derived from octopine strains were ineffective (Fry *et al.* 1987; Pua *et al.*, 1987). Marked differences among *B. napus* genotypes in relation to shoot regeneration have been demonstrated (Khehra and Mathias, 1992). Also the choice of target tissue for *A. tumefaciens* transformation has been shown to be of great importance. Successful results for transformation of *B. napus* have been obtained with target tissue from various parts of the plant such as internodes (Fry *et al.*, 1987), stems (Pua *et al.*, 1987), thin cell layers (Charest *et al.*, 1988), hypocotyls (Radke *et al.*, 1988; De Block *et al.*, 1989) and cotyledons (Moloney *et al.*, 1989).

The majority of transformation studies on *B. napus* has been performed with spring cultivars, and the genotypes have largely been restricted to a few cultivars, with cv. Westar the predominant genotype. Previous publications describing transformation and regeneration protocols for rapeseed have emphasised the limitations in the regeneration potential of rapeseed winter cultivars. De Block *et al.* (1989) described a transformation protocol for a single winter cultivar of *B. napus* (R8494) by use of two selection genes: the *bar* gene, coding for an enzyme inactivating the herbicide phosphinothricin; and the *neo* gene, conferring resistance to kanamycin. Using a binary *A. rhizogenes* vector, Boulter *et al.* (1990) obtained transgenic shoots from hairy root cultures of two spring cultivars and the winter cultivar Bienvenue. Approximately 1% of the Bienvenue hairy root clones produced shoots. Using *A. tumefaciens*, Boulter *et al.* (1990) also obtained transgenic plants from two spring cultivars and the winter cultivar Cobra. The transformation rate for the winter cultivar Cobra was only 25% of that of the spring cultivar Westar. Also Stefanov *et al.* (1994) reported a lower transformation rate of two *B. napus* winter cultivars, Santana and Arabella, compared to the spring cultivar Hanna. These rather low frequencies of transformants and large genotypic variations are still the main obstacle in transformation of *B. napus* winter cultivars. However, the potential use of genetic transformation as a method to improve qualitative and quantitative yield factors in rapeseed winter cultivars, e.g. resistance to pathogens or herbicides should be emphasized. From an agronomical and an environmental point of view, initiatives in growing winter cultivars of *Brassica napus* have increased during recent years due to the higher yield of winter crops, and due to the capability of winter crops to utilize excess nitrogen fertilizer in the fields during the winter period.

The aim of the present study was to establish an efficient and reproducible transformation method for a number of rapeseed winter cultivars. We describe here the generation of transgenic plants from six commercial *B. napus* winter cultivars by use of two different inoculation methods and by use of five *A. tumefaciens* strains, comprising combinations of the selection genes NPTII and HPT and the dominant GUS reporter gene. The genotype-dependent capability for regeneration of transgenic plants and the stable inheritance of the marker genes to the F_1 generation after self-pollination was demonstrated.

Materials and methods

PLANT MATERIAL

Seeds of *B. napus* were soaked in 70% EtOH for 1 min, surface-sterilized for 30 min in a 7.5% Ca-hypochlorite solution containing 0.1% "Tween-20", rinsed three times with sterile distilled water and germinated on hormone-free MS medium (Murashige and Skoog, 1962) at 25°C in continuous light, 150 $\mu E\,m^{-2}\,s^{-1}$ (light source Phillips TL-33, 36W).

BACTERIAL STRAINS

Five disarmed *A. tumefaciens* strains were used throughout this study (bacterial selection markers are listed in brackets): two nopaline strains, C58C1pGV3805p-GSGLUC1 (carbenicillin, spectinomycin), C58C1pMP90p-Bil21 (gentamycin, kanamycin) (both strains were kindly donated by Plant Genetic Systems, NV, Gent, Belgium), two octopine strains, LBA4404pAL4404pBi121 (streptomycin, kanamycin) (a kind gift from Dr G. Ooms, Rothamsted Experimental Station, UK) and LBA4404-pAL4404pJIT73 (carbenicillin, kanamycin) (a kind gift from Dr Mullineaux, John Innes Institute, Norwich, UK) and a strain C58C1pGV2260pBil21 (carbenicillin, kanamycin) constructed in our laboratory encompassing a nopaline chromosomal background and an octopine-derived virulence plasmid.

The binary plasmids in the *A. tumefaciens* strains were derived from various origins. The 14 kb pGSGLUC1 plasmid contains the NPTII gene (encoding neomycin phosphotransferase), and the GUS (β-glucuronidase) reporter gene. These genes were regulated by the dual, bi-directional mannopine synthase promoter PTR1'-2' (Velten and Schell, 1985). The pBi121 is a 13 kb plasmid harbouring the NPTII and the GUS genes (Jefferson *et al.*, 1987). The NPTII gene (Herrera-Estrella *et al.*, 1983) was controlled by the nopaline synthase promoter (Depicker *et al.*, 1982) and the GUS gene was driven by the CaMV 35S promoter (Odell *et al.*, 1985). The 17.5 kb plasmid pJIT73 encompasses the NPTII gene and the GUS gene and in addition the HPT gene (encoding hygromycin phosphotransferase) (Waldron *et al.*, 1985) driven by the CaMV 35S promoter. All *A. tumefaciens* strains were grown in LB medium at 28°C and with 50 $mg\,l^{-1}$ of each of the two bacterial selectable antibiotics (for streptomycin 500 mg^{-1} was used). An overnight culture was harvested by centrifugation at 5000 × g for 10 min. To avoid residues of antibiotics

the pellet was washed twice in sterile water and finally resuspended in an appropriate medium.

TRANSFORMATION, SELECTION AND REGENERATION

For transformation of *B. napus* two inoculation methods were studied.

Inoculation method A. This method was accomplished according to Moloney *et al.* (1989) with minor modifications. Cotyledons from 7-day-old seedlings were excised and inoculated with one of the *A. tumefaciens* strains (Table 1) at the cut surface of the petiole. An inoculation medium MS-MES medium (4.6 g l^{-1} MS basal salt mixture including vitamins (Duchefa, Haarlem, The Netherlands) in 2.5 mM MES buffer, pH 5.5) with 200 µM acetosyringone, 1 mg l^{-1} 2,4D (2,4-dichlorophenoxyacetic acid) and 1% (w/v) agarose (Sea Plaque, low gelling temperature) was autoclaved and chilled to 30°C. An *A. tumefaciens* culture grown over night was adjusted to a density of $OD_{600nm} = 1$. After centrifugation the pellet was washed twice with MS-MES medium, resuspended in 18 ml MS-MES medium and mixed with 2 ml inoculation medium. This bacterial suspension was taken up into a 20 ml syringe and droplets, approximately 40 µl, were placed with 1 cm distance on a Wattman 3MM filter paper wetted with MS medium in a 9 cm Petri dish. Excised cotyledons were placed with the cut surface in the droplets and inoculated in continuous light (150 µE m^{-2} s^{-1}) at 25°C for 7 days. Then the petioles and the cotyledons were separated and the petioles were transferred to a callus inducing medium with the same composition as the inoculation medium but with 50 mg l^{-1} kanamycin (Table 1) and without acetosyringone. After one week the callus tissue was transferred to a shoot inducing medium (MS-MES medium, 20 g l^{-1} sucrose, 40 mg l^{-1} adenine, 1 mg l^{-1} BAP (benzylaminopurine), 0.1 mg l^{-1} NAA (naphthalene-acetic acid), 0.01 mg l^{-1} GA_3 (gibberellic acid), 500 mg l^{-1} PVP (polyvinylpyrolidone), 5 mg l^{-1} silver nitrate, 20 mg l^{-1} kanamycin, 5 g l^{-1} agarose and 250 mg l^{-1} carbenicillin). Selection of transformed shoots was accomplished over a period of three weeks. Excised shoots were then transferred to a rooting medium (MS-MES medium, 7 g l^{-1} agarose).

Inoculation method B. This method was performed according to protocol described by De Block *et al.* (1989) with minor modifications. The washed *A. tumefaciens* (LBA4404pAL4404pJIT73) pellet was resuspended in a sucrose free cocultivation medium (MS-MES medium, pH 5.5, 1 mg l^{-1} BAP, 0.1 mg l^{-1} NAA, 0.01 mg l^{-1} GA_3, 200 µM acetosyringone). The bacterial suspension was adjusted to a final density of $OD_{600nm} = 1.0$. Hypocotyls, isolated from 14-day-old seedlings, were cut into 10 mm segments, placed in 9 cm Petri dishes and covered with the bacterial suspension. After 30 min the bacterial suspension was removed and the hypocotyl segments were transferred to sucrose-free cocultivation medium solidified with 0.8% agar. 50 hypocotyl segments were placed on 20 ml medium in 9 cm Petri dishes and kept for 5 days in darkness at 21°C. Then the explants were transferred to a callus-inducing medium (MS-MES medium, pH 5.7, 30 g l^{-1} sucrose, 1 mg l^{-1} kinetin, 1 mg l^{-1} 2, 4D, 0.01 mg l^{-1} GA_3, 500 mg l^{-1} PVP, 5 mg l^{-1} $AgNO_3$, 5 g l^{-1} agarose, 250 mg l^{-1} carbenicillin) and kept at 25°C in continuous light (150 µE m^{-2} s^{-1}). After 2 weeks, callus emerged at the cut surfaces of the hypocotyl segments. The explants were now transferred to a selective, shoot inducing medium as in method A but with 30 mg l^{-1} hygromycin as selecting agent (Table 1). Approximately five weeks after termination of cocultivation, small dark-green shoot meristems emerged on the calluses. Within a period of 12 weeks after cocultivation was finished, new shoot meristems were formed. The explants with the shoot primordia were transferred to 350 ml jam glasses with 30 ml shoot elongation medium (MS-MES medium pH 5.7, 10 g l^{-1} sucrose, 0.0025 mg l^{-1} BAP, 30 mg l^{-1} hygromycin and 7 g l^{-1} agarose). After 2–3 weeks, emerging shoots were excised and transferred to rooting medium.

Table 1. *Agrobacterium tumefaciens* strains used for transformation of *B. napus* winter cultivars. Concentrations of antibiotics used for selection of transgenic tissue during regeneration, a: callus inducing medium, b: shoot inducing medium, c: root inducing medium. kan: kanamycin, hyg: hygromycin

A. tumefaciens strains	*Chromosomal background*	*Virulence plasmid*	*Inoculation method*		*T-DNA*		
					a (mg l^{-1})	*b* (mg l^{-1})	*c* (mg l^{-1})
C58C1pGV3850pGSGLUC1	nopaline	nopaline	A	kan	50	20	0
C58C1pMP90pBi121	nopaline	nopaline	A	kan	50	20	0
C58C1pGV2260pBi121	nopaline	octopine	A	kan	50	20	0
LBA4404pAL4404pBi121	octopine	octopine	A	kan	50	20	0
			B	kan	100	100	0
LBA4404pAL4404pJIT73	octopine	octopine	B	hyg	0	30	0

HISTOCHEMICAL GUS ASSAY

Assay for histochemical GUS activity in putative transgenic plants was carried out on the uppermost leaf according to the method of Kosugi *et al.* (1990). GUS activity in the leaf tissue was identified after an overnight incubation at 37°C in a reaction mixture containing 50 mM sodium-phosphate buffer pH 7.0, 10 mM EDTA, 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-glucuronide), 20% w/v methanol.

SOUTHERN BLOT ANALYSIS

Total DNA from GUS$^+$ plants and from non-transformed control plants (wt) was isolated from leaves of 4-6 weeks old plants and purified by CTAB precipitation according to the method of Dellaporta *et al.* (1984). After endonuclease digestion with either *Hind*III, *Nco*I or *Bgl*II (Amersham International Inc., Little Chalfont, UK) and separation on a 0.7% (w/v) agarose gel (Bio-Rad Laboratories, Hercules, CA, USA) DNA was transferred to Zeta Probe GT membranes (Bio-Rad). Filters were hybridized in 50% formamide at 42°C in a Hybaid Hybridization Oven and subsequently exposed to Fuji Medical X-ray films for 14 days.

PROBES

Three probes, specific for the HPT, NPTII and the GUS genes were used in the Southern blot analysis. The HPT probe was isolated as an internal *Ksp*I-*Eco*R1 530 bp fragment from the plasmid pTAD7 (De Block and Debrouwer, 1991). The NPTII probe was isolated as an internal 433 bp *Nco*I-*Nar*I fragment from the plasmid pGSGLUC1. The GUS probe was derived as an internal 573 bp *Bam*HI-*Eco*RV fragment from the plasmid pBi121 (Jefferson *et al.*, 1987). The probes were radiolabelled with α-^{32}P dATP (Amersham) using a Random Oligolabelling Kit (Boehringer).

VERNALIZATION

The rapeseed winter cultivars were induced to flowering by vernalization at 4°C for 12 weeks at low light intensity (50 $\mu E\,m^{-2}\,s^{-1}$). The plants were brought to flowering and self-pollinated in a growth chamber under 16 h light (800 $\mu E\,m^{-2}\,s^{-1}$) and a 22/18°C day/night temperature regime. Cross-pollination was avoided by covering the inflorescence with plastic bags.

PROGENY STUDIES

In a test seeds from two transgenic *B. napus* cv. Falcon plants were germinated and grown in the growth chamber. Three F_1 plants, F73-1, F73-2, F73-3, derived from the R_0 parent plant F73 and two F_1 plants, F122-3, F122-4, derived from the R_0 parent plant F122, were subjected to Southern blot analyses to test the stability of the integrated T-DNA copies.

Results

REGENERATION OF TRANSGENIC PLANTS

Genetic transformation of six winter cultivars and two spring cultivars of *B. napus* was performed using five strains of *A. tumefaciens* harbouring different combinations of chromosomal backgrounds and virulence plasmids from octopine and nopaline strains as listed in Table 1. Two inoculation methods of the explants were applied. In method A, petioles of excised 7-day-old cotyledons were placed in droplets of an *A. tumefaciens* suspension, solidified by agarose. Cocultivation spanned 7 days. In method B, hypocotyl segments were cocultivated with *A. tumefaciens* for 5 days. Developmental stages of regenerated plants from both methods are shown in Fig. 1. The target tissue at the cut cotyledon petiole surface expanded considerably during the 7-day cocultivation period (Fig. 1a). Histochemical GUS activity could be detected in the target tissue 2-3 weeks after inoculation and in regenerated transgenic shoots 10-12 weeks after inoculation (Fig. 1b). GUS activity in callus tissue at the cut surface of hypocotyl segments of cv. Falcon, (method B), three weeks after inoculation is demonstrated in Fig. 1c. A fully developed transgenic plant of *B. napus* cv. Falcon compared to a non-transformed control plant is shown in Fig. 1d. No differences in the phenotype of the transgenic plants compared to the non-transformed plants were detected. A flowering transgenic plant of cv. Falcon is shown in Fig. 1e. Flowering was induced by a 12-week vernalization period at 4°C. The vernalization requirement for flowering was not affected by the passage through the tissue culture phase in any of the tested winter cultivars. No morphological abnormalities in the transgenic plants during flowering and fruit setting were detected. Seeds from 25 transgenic *B. napus* plants, representing the three winter cultivars, cv. Accord, cv. Falcon and cv. Libraska, were collected for further analysis.

The total score of transgenic plants, obtained from six winter cultivars and two spring cultivars by the two inoculation methods A and B and based on the histochemical GUS assay, is shown in Table 2. The transformation efficiency of the five *A. tumefaciens* strains and the capability in regeneration of transgenic plants of the six winter cultivars showed great variability. Using the nopaline strains C58C1pGV3850pGSGLUC1 and C58C1pMP90pBi121, encompassing nopaline chromosomal backgrounds and nopaline virulence plasmids, a score between 6–16% GUS$^+$ plants was obtained. Using the strain C58C1pGV2260pBi121, harbouring a combination of a nopaline chromosomal background and an octopine virulence plasmid, a low range of transformation, between 0–4%, was scored. Of the two octopine strains LBA4404pAL4404pBi121 and LBA4404pAL4404-pJIT73, harbouring both an octopine chromosomal background and an octopine virulence plasmid, only pJIT73

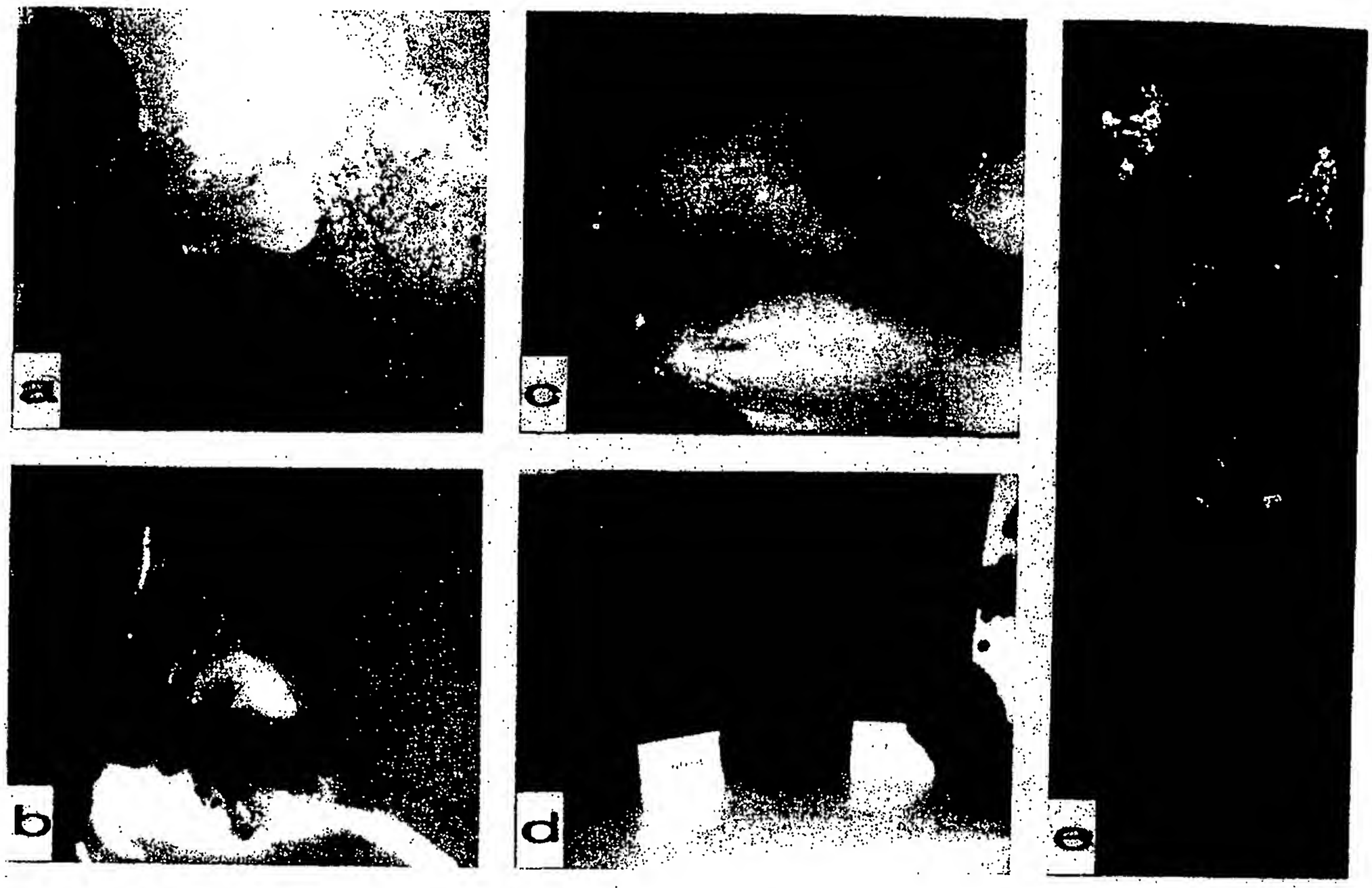

Fig. 1. Development of transgenic plants from two *B. napus* winter cultivars cv. Accord and cv. Falcon. (a) Excised cotyledons from *B. napus* cv. Accord used as target tissue for inoculation with *A. tumefaciens* pGSGLUC1 (method A). After cocultivation for seven days callus formation was visible at the cut surface of the petiole. (b) A GUS$^+$ shoot (blue colour) of *B. napus* cv. Accord, regenerated on a kanamycin containing medium twelve weeks after inoculation. The light areas on the explant are non-transformed dead callus tissue. (c) Hypocotyl segments of *B. napus* cv. Falcon transformed with *A. tumefaciens* LBA4404pAL4404pJIT73 (method B). GUS activity in the tissue at the cut surface of the segments is demonstrated by use of the X-Gluc assay three weeks after inoculation. (d) A fully developed GUS$^+$ plant of *B. napus* cv. Falcon (right) and a non-transformed control plant (left). (e) Inflorescent stalk of a GUS$^+$ cv. Falcon plant. Flowering began two weeks after a 12-week vernalization period.

Table 2. Six *B. napus* winter cultivars and two spring cultivars transformed with five *A. tumefaciens* strains by use of two inoculation methods, A and B. The number of explants and the number of GUS$^+$ plants (the frequency in % is shown in brackets) for each transformation experiment are shown.

Inoculation method	*A*		*A*		*A*		*A*		*B*	
A. tumefaciens strain	*C58C1, pGV3850pGSGLUC1*		*C58C1, pMP90pBi121*		*C58C1, pGV2260pBi121*		*LBA4404, pLBA4404pBi121*		*LBA4404, pAL4404pJIT73*	
	No. expl.	*GUS$^+$ plants*	*No. expl.*	*GUS$^+$ plants*	*No. expl.*	*GUS$^+$ plants*	*No. expl.*	*GUS$^+$ plants*	*No. expl.*	*GUS$^+$ plants*
B. napus cv										
Accord*	212	27 (13%)	152	9 (6%)	151	4 (3%)	178	0 (0%)	400	1 (0%)
Bienvenue*	150	9 (6%)	56	10 (6%)	57	2 (4%)	157	0 (0%)	nt	
Cobra*	132	11 (8%)	242	35 (14%)	109	4 (4%)	189	1 (0%)	nt	
Libraska*	137	13 (10%)	183	31 (16%)	70	2 (3%)	113	5 (4%)	151	2 (2%)
Falcon*	182	17 (9%)	99	6 (6%)	38	0 (0%)	68	0 (0%)	500	61 (12%)
Zeus*	112	3 (3%)	nt		nt		nt		275	3 (1%)
Line**	120	8 (7%)	nt		nt		nt		250	20 (8%)
Westar**	99	12 (12%)	nt		nt		nt		250	12 (4%)

*winter cultivar
**spring cultivar

revealed a high transformation rate (12%) and only in combination with cv. Falcon. Three other *B. napus* winter cultivars, cv. Ceres, cv. Carina, and cv. Jupiter were inoculated with LBA4404pAL4404pJIT73 using inoculation method B. They all revealed frequencies of 0–1% transgenic shoots (data not shown). For comparison the transformation frequency of the two *B. napus* spring cultivars cv. Line and cv. Westar was tested. Transgenic shoots within the range of 4–12% were obtained.

Kanamycin was used as the only selecting agent in the regeneration of plants from method A. Selection of plants regenerated from method B was performed with either kanamycin or hygromycin and here hygromycin turned out to be more efficient than kanamycin. The concentrations of antibiotics used for selection during the callus phase and the shoot-inducing phase are listed in Table 1. The lower limits for selection of transgenic explants were 20 mg l^{-1} kanamycin or 10 mg l^{-1} hygromycin. Lower concentrations of antibiotics caused an increase in the number of escapers. On the other hand if the concentrations of selecting antibiotics were increased above the levels listed in Table 1, an inclination to vitrification of the regenerated plants was detected. At a concentration of either 100 mg l^{-1} kanamycin or 30 mg l^{-1} hygromycin in the shoot inducing medium, the number of escapers did not exceed 10% (data not shown). Silver nitrate, 5 mg l^{-1} was required for shoot regeneration. Carbenicillin was added to prevent silver chloride precipitation (De Block *et al.*, 1989).

HISTOCHEMICAL ANALYSIS OF GUS EXPRESSION

Histochemical GUS assays were performed on leaf tissue in order to select transgenic plants. The number of detected GUS$^+$ plants are listed in Table 2. No intrinsic GUS activity was detected in any of the non-transformed rapeseed cultivars analysed in this study.

SOUTHERN HYBRIDIZATION ANALYSIS

In order to confirm stable integration of T-DNA representative GUS$^+$ *B. napus* plants from the two transformation combination C58C1pGV3850pGSGLUC1/cv. Accord and LB4404pAL4404pJIT73/cv. Falcon were investigated by Southern blot analysis (Figs 2 and 3). Leaf DNA, isolated from one cv. Accord plant, A10, was cut with either *Hind*III (Fig. 2a) or *Nco*I (Figs 2b, c). The DNA was probed with a radiolabelled internal fragment of either the GUS gene (Figs 2a, b) or with the NPTII gene (Fig. 2c). When hybridized to the 570 bp GUS probe the DNA, digested with *Hind*III, revealed one single band of 4.9 kb. Also plant DNA, digested with *Nco*I, revealed one single band of 4.8 kb. When the *Nco*I digested DNA was hybridized to the NPTII probe two bands of 6.0 kb and 4.8 kb were detected (Fig. 2c). The appearance of one hybridization band (Figs 2a, b) indicates the integration of only one copy of T-DNA into the genome of A10. The

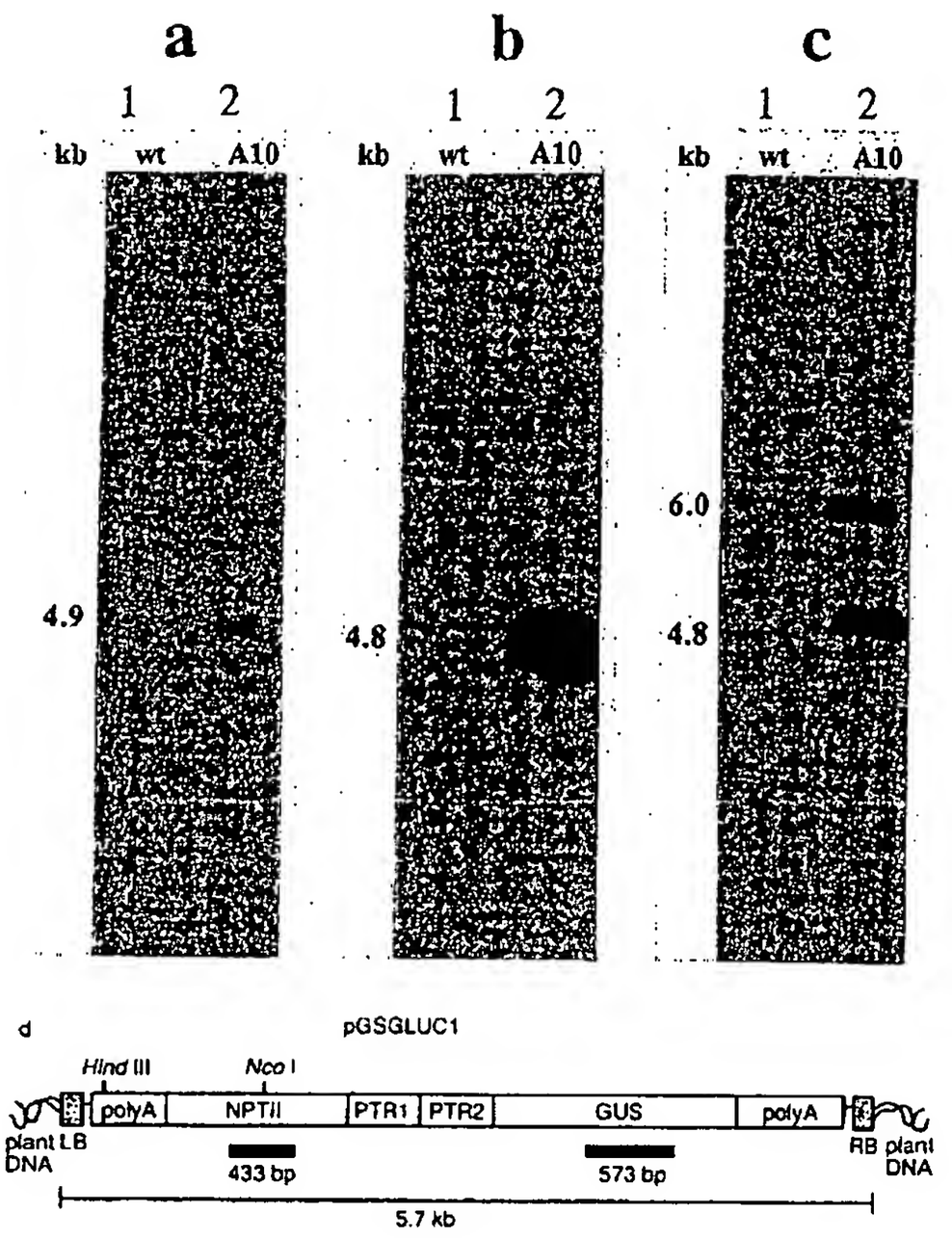

Fig 2. Southern blot analysis of DNA isolated from leaves of *B. napus* cv. Accord transformed by use of *A. tumefaciens* C58C1pGV3850pGSGLUC1. DNA from a control plant (wt) and from a GUS$^+$ plant, A10, were digested with either *Hind*III or *Nco*I and probed with radiolabelled internal fragments of the GUS or the NPTII coding sequences: (a) DNA digested with *Hind*III and probed with GUS; (b) DNA digested with *Nco*I and probed with GUS; (c) DNA digested with *Nco*I and probed with NPTII. Sizes in kb of the hybridized fragments are shown to the left of the panels. A schematic diagram of the T-DNA region of the plasmid pGSGLUC1, integrated into the plant DNA, and the localization of the NPTII probe (433 bp) and the GUS probe (573 bp) are shown in (d). Symbols, LB: T-DNA left border, RB: T-DNa right border, NPTII: kanamycin resistance gene. GUS: β-glucuronidase gene, polyA: 3′ polyadenylation sequence. PTR1/PTR2: dual bidirectional mannopine synthase promoter.

appearance of two hybridization bands in the *Nco*I/NPTII combination is in accordance with the position of the *Nco*I restriction site within the sequence of the 433 bp NPTII probe (Fig. 2d). DNA from a non-transformed cv. Accord plant (wt) did not show any hybridization signals.

Southern blot analysis of three GUS$^+$ plants of *B. napus* cv. Falcon is shown in Fig. 3. DNA from the plants F73, F122, F144 was digested with the restriction enzyme *Bgl*II and hybridized to either the HPT probe

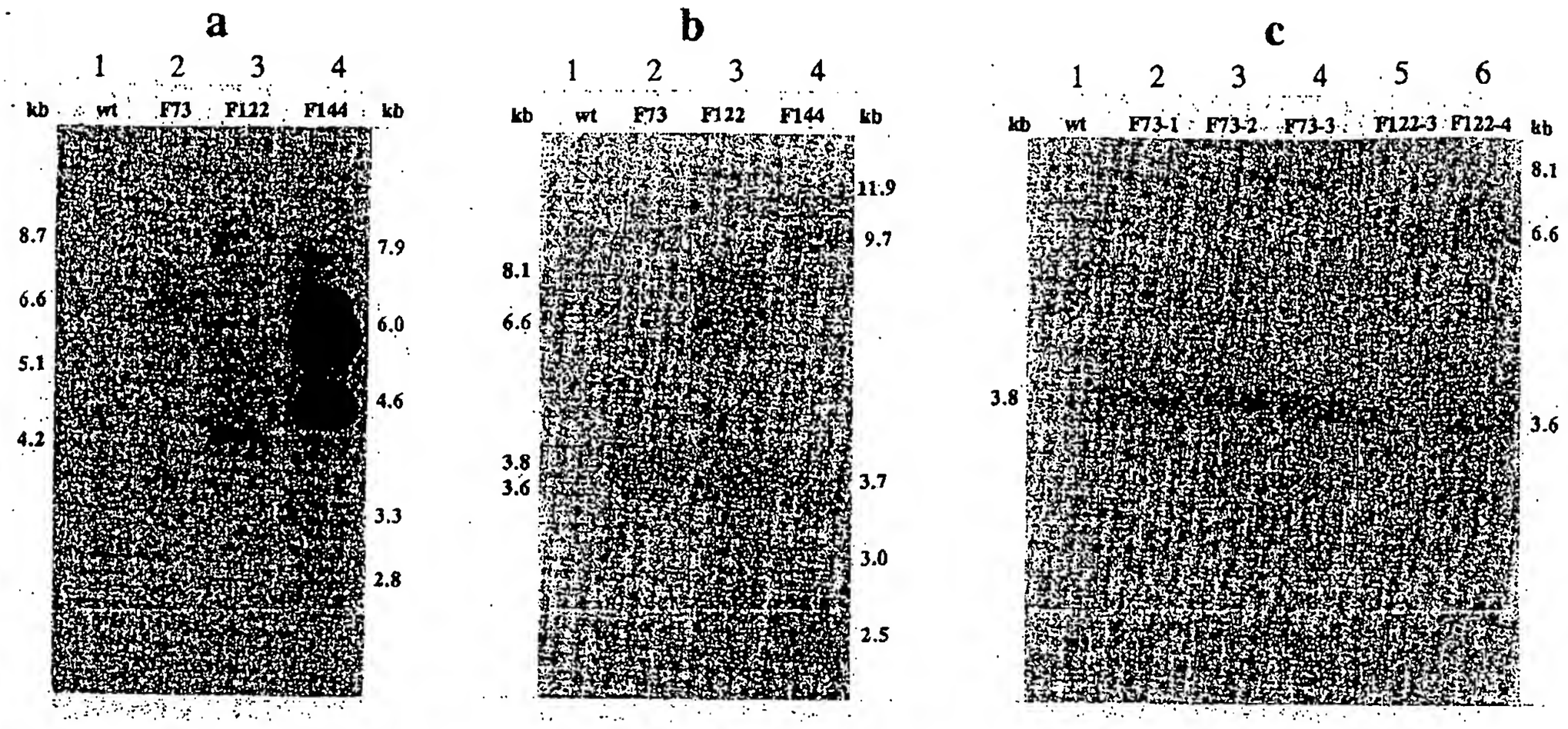

Fig 3. Southern blot analysis of DNA isolated from leaves of *B. napus* cv. Falcon transformed by use of *A. tumefaciens* LBA4404pLBA4404pJIT73. DNA was digested with the *Bgl*II restriction enzyme. Digested DNA from control plants (wt) and three GUS$^+$ plants, F73, F122 and F144, derived from three individual transformation events, were probed with radiolabelled 530 bp internal fragment of the HPT coding sequence (a) and with radiolabelled 433 bp internal fragment of the NPTII coding sequence (b) After self-pollination and fruit setting seeds were collected and germinated. DNA from leaves of GUS$^+$ F_1 plants, digested with *Bgl*III, were subjected to Southern blot analysis and probed with NPTII fragment. Three representative F_1 plants, F73-1, F73-2 and F73-3, originated from the R_0 parent F73 and two F_1 plants, F122-3, F122-4 originated from the R_0 parent F122 were analysed (c) A schematic diagram of the T-DNA region of the plasmid pJIT73, integrated into the plant DNA, and the localization of the NPTII probe HPT (530 bp) and NPTII (433 bp) are shown. (d) Symbols, HPT; hygromycin resistance gene, 35S: CaMV 35S promoter Pnos: nopaline synthase promoter, other symbols as in Fig. 2.

(Fig. 3a) or the NPTII probe (Fig. 3b). Integration of different numbers of T-DNA copies was observed, ranging from one copy in F73, three copies in F122 to five copies in F144 when hybridized with the HPT probe (Fig. 3a) and with the NPTII probe (Fig. 3b). A weak unspecific 7.2 kb hybridization band was observed when HPT probe was used (Fig. 3a) indicating a weak cross-hybridization to an endogenous plant sequence. However, the Southern blot analysis with the same DNA digest and hybridization to the NPTII probe did not reveal any non-specific hybridization bands (Fig. 3b).

F_1 seeds harvested after self-pollination of the two R_0 plants, F73 and F122 were germinated and tested for histochemical GUS activity. Representatives of the GUS$^+$ F_1 plants were subjected to Southern blot analysis and probed with the NPTII fragment (Fig. 3c). This analysis showed that F_1 plants from F73 contained one T-DNA copy, illustrated by the 3.8 kb hybridization band. Of the two F_1 plants from F122, F-122-3 contained two of the parental T-DNA copies, illustrated by the 6.6 and 3.6 kb hybridization bands, while F122-4 in addition to these two bands also contained the 8.1 kb parental band. The size of the hybridization bands in the F_1 plants was exactly the same as in the respective R_0 plants, providing strong evidence that the T-DNA copies are stably integrated into the plant genome. In Fig. 2d it is illustrated that when the plant DNA is digested with either *Hind*III or *Nco*I the GUS probe will hybridize to

the T-DNA/plant DNA junction including the RB border region. When plant DNA was digested with *Hind*III the NPTII probe hybridized to T-DNA/plant DNA junction including the RB border region, while digestion with *Nco*I and hybridization with NPTII revealed two T-DNA/plant junctions including either the RB region or the LB border region.

Further analysis of the segregation of the marker genes in the F_1 progenies of the transgenic plants is in progress.

Discussion

Methods for efficient genetic transformation of winter cultivars of *B. napus* with binary nopaline and octopine derived strains of *A. tumefaciens* were studied. The rapeseed winter cultivars cv. Accord, cv. Cobra, cv. Falcon and cv. Libraska yielded a transformation efficiency of 13%, 14%, 12% and 16%, respectively (Table 2). These transformation frequencies are amongst the highest reported for winter cultivars of *B. napus*. In a recent transformation experiment with two *B. napus* winter cultivars, cv. Santana and cv. Arabella, Stefanov *et al.* (1994) recovered 4% and 3% GUS-positive plants, respectively by use of the octopine strain LBA4404-pIS412, harbouring the Pnos/GUS construction. By use of a nopaline strain, C58C3pJIT73 Boulter *et al.* (1990) obtained 3 GUS-positive shoots from 91 shooting explants of the *B. napus* winter cultivar cv. Cobra.

A survey in the literature of *A. tumefaciens* vectors used for transformation of *B. napus* revealed that octopine strains were generally reported to be less effective in this process compared to nopaline strains (Holbrook and Miki, 1985; De Block *et al.* 1989). However, in other studies (Fry *et al.*, 1987; Charest *et al.*, 1988) susceptibility of *B. napus* to engineered as well as wild-type octopine strains was reported. In the present study transgenic plants were achieved by transformation with the nopaline strains, C58C1pGSGLUC1 and C58p-MP90pBi121 as well as the octopine strain LBA4404p-Al4404pJIT73. Successful transformation with the latter was obtained with only one *B. napus* cultivar, cv. Falcon.

In our studies addition of acetosyringone (200 μM) to the inoculation medium was a prequisite for positive transformation. This finding is in keeping with Charest *et al.* (1988) who stated that in experiments with transformation of *B. napus* by use of octopine strains the *vir* region should be activated by either acetosyringone or wounded cells from tobacco leaf discs. In the present study, the binary plasmid pBi121 was used with three different combinations of virulence plasmids and chromosomal background (Table 1). The highest rate of transformation with pBi121 was obtained when this plasmid was used in combination with the supervirulent plasmid pMP90.

Selection of target tissue at an optimal physiological and developmental stage is important for *A. tumefaciens*-mediated transformation. In our experiments, inoculation of 7-day-old cotyledons (method A) revealed a high transformation rate compared to inoculation of hypocotyl segments (method B). The extensive expansion of the callus tissue at the cut surface of the cotyledon petiole (Fig. 1a) may be essential for a successful transformation, since only a limited number of cells are competent for both transformation and regeneration. We observed that regeneration of shoots from cotyledon petioles (method A) was faster than regeneration of shoots from hypocotyl segments (method B), which implies that the period of callus growth and the risk of undesired somaclonal variations is reduced. We demonstrate for the first time transformation of *B. napus* winter cultivars using cotyledon petioles as target tissue. Compared to hypocotyls, transformation of cotyledon explants was shown to be genotypically independent for cultivars tested. In hypocotyl segments no cell division activity and callus formation was observed during the cocultivation period. The extensive cell division activity in the cotyledon explants during the cocultivation period may promote cells with a higher capability for incorporation of T-DNA into the plant genome and regeneration to intact plants.

Difficulties have been experienced by some authors in using kanamycin as selective antibiotic in production of transgenic rapeseed shoots (Pua *et al.*, 1987). In *B. napus* winter cultivars we found that hygromycin as well as kanamycin could be used as selective agents. The optimal concentration of antibiotics during regeneration of shoots from hypocotyl segments (method B) was in average 2 to 3 times higher for kanamycin than for hygromycin.

In this study, all putative transgenic plants that passed the antibiotic selection procedure during regeneration were analysed for GUS activity by use of the histochemical X-Gluc test. Selected GUS^+ plants were subjected to Southern blot analysis to confirm the presence of incorporated T-DNA (Figs 2 and 3). One cv. Accord plant, A10, (Fig. 2) and three cv. Falcon plants, F73, F122 and F144 (Fig. 3) were analysed. Care was taken to apply the same amount of DNA to the gels before electrophoresis. It should be emphasized that the restriction sites shown on the maps (Figs 2d and 3d) were strictly coordinated to the position of the HPT probe and the NPTII probe. Each of the two probes was located within a DNA fragment spanning the end part of the T-DNA and part of the plant DNA. This particular location of the probes revealed the number of individual T-DNA integrations in the plant genome. Therefore it is likely that the plant A10 possessed one copy and the three plants, F73, F122 and F144 possessed one, three and five integrated T-DNA copies, respectively. In F144 two bands, 6.0 kb and 4.6 kb (Figs 3a, lane 4) revealed a stronger hybridization signal compared to the general level of hybridization intensity. A putative interference

with contaminating *Agrobacterium* plasmid DNA in the transgenic plants was tested and can be excluded due to the size of these fragments (data not shown). The size of a direct repeated integration does not correspond to the size of any of the bands. Integration of an inverted repeat including two LB orders could account for the 4.6 kb band. However, the finding of five hybridization bands in F144 probed with the HPT probe as well as the NPTII probe excludes the integration of an inverted tandem repeat. Differences in hybridization signals within the same transgenic rapeseed plant have been demonstrated by other authors (Radke *et al.*, 1988; Moloney *et al.*, 1989).

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References

Altenbach, S.B., Kuo, C.C., Staraci, L.C., Pearson K.W., Wainwright, C., Georgescu, A. and Townsend, J. (1992) Accumulation of a Brazil nut albumin in seeds of transgenic canola results in enhanced levels of speed protein methionine. *Plant Mol. Biol.* **18**, 235–46.

Boulter, M.E., Croy, E., Simpson, P., Shields, R., Croy, R.R. D. and Shirsat, A.H. (1990) Transformation of *Brassica napus* (oilseed rape) using *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* – a comparison. *Plant Sci.* **70**, 91–9.

Charest, P.J., Holbrook, L.A., Gabard, J., Iyer, V.N. and Miki, B.L. (1988) *Agrobacterium*-mediated transformation of thin cell layer explants from *Brassica napus* L. *Theor. Appl. Genet.* **75**, 438–45.

Chen, J.L. and Beversdorf, W.D. (1994) A combined use of microprojectile bombardment and DNA imbibition enhances transformation frequency of canola *(Brassica napus* L.). *Theor. Appl. Genet.* **88**, 187–92.

Damgaard, O., and Rasmussen, O. (1991) Direct regeneration of transformed shoots in *Brassica napus* from hypocotyl infections with *Agrobacterium rhizogenes*. *Plant Mol. Biol.* **17**, 1–8.

De Block, M., Debrouwer, D. and Tenning, P. (1989) Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in the transgenic plants. *Plant Physiol.* **91**, 694–701.

De Block, M. and Debrouwer, D. (1991) Two T-DNA's cotransformed into *Brassica napus* by double *Agrobacterium tumefaciens* infection are mainly integrated at the same locus. *Theor. Appl. Genet.* **82**, 257–63.

Dellaporta, S.L., Wood, J., and Hicks, J.B. (1984) A plant DNA minipreparation. In: *A Laboratory Course Manual*, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, pp. 36–7.

Depicker, A., Stachel, S., Dhaese, P., Zambryski, P. and Goodman, H.M. (1982) Nopaline synthase: Transcript mapping and DNA sequence. *J. Mol. Appl. Genet.* **1**, 561–73.

Downey, R.K. and Röbbelen, G. (1989) *Brassica* species. In: Röbbelen, G., Downey, R. K. and Ashri, A. eds, *Oil Crops of the World*, New York, NY: McGraw-Hill Inc., pp. 339–62.

Fry, J., Barnason, A. and Horsch, R.B. (1987) Transformations of *Brassica napus* with *Agrobacterium tumefaciens* based vectors. *Plant Cell Rep.* **6**, 321–5.

Herrera-Estrella, L., De Block, M., Messens, E., Hernalsteens, J.P., Van Montagu, M. and Schell, J. (1983) Chimeric genes as dominant selectable markers in plant cells. *EMBO J.* **2**, 987–95.

Hollbrook, L.A. and Miki, B.L. (1985) *Brassica* crown gall tumourigenesis and *in vitro* culture of transformed tissue. *Plant Cell Rep.* **4**, 329–32.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: β-glucuronidase as a sensitive and versaltile gene fusion marker in higher plants. *EMBO J.* **6**, 3901–7.

Khehra, G.S. and Mathias, R.J. (1992) The interaction of genotype, explant and media on the regeneration of shoots from complex explants of *Brassica napus* L. *J. Exp. Bot.* **43**, 1413–8.

Knutzon, D.S., Thompson, G.A., Radke, S.E., Johnson, W.B., Knauf, V.C. and Kridl, J.C. (1992) Modification of *Brassica* seed oil by antisense expression of a stearoyl-acyl carrier protein desaturase gene. *Proc. Natl Acad. Sci. USA* **89**, 2624–8.

Kosugi, S., Ohashi, Y., Nakajima, K. and Arai, Y. (1990) An improved assay for β-glucuronidase in transformed cells: Methanol almost completely suppresses a putative endogenous β-glucuronidase activity. *Plant. Sci.* **70**, 133–40.

Moloney, M.M., Walker, J.M. and Sharma, K.K. (1989) High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. *Plant. Cell Rep.* **8**, 238–42.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–97.

Odell, J.T., Nagy, F. and Chua, N.H. (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature* **313**, 810–2.

Pua, E.C., Mehra-Palta, A., Nagy, F. and Chua, N.H. (1987) Transgenic plants of *Brassica napus* L. *Bio/Technology* **5**, 815–7.

Radke, S.E., Andrews, B.M., Moloney, M.M., Crouch, M.L., Kridl, J.C. and Knauf, V.C. (1988) Transformation of *Brassica napus* L. using *Agrobacterium tumefaciens*: developmentally regulated expression of an reintroduced napin gene. *Theor. Appl. Genet.* **75**, 685–94.

Rasmussen, J. and Rasmussen, O.S. (1993) PEG mediated DNA uptake and transient GUS expression in carrot, rapeseed and soybean protoplasts. *Plant Sci.* **89**, 199–207.

Schröder, M., Dixelius, C., Råhlén, L. and Glimelius, K. (1994) Transformation of *Brassica napus* by using the *aadA* gene as selectable marker and inheritance studies of the marker genes. *Physiol. Plant.* **92**, 37–46.

Sjödin C. (1992) Brassicaceae, a plant family well suited for modern biotechnology. *Acta. Agric. Scand.* **42**, 197–207.

Spangenberg, G., Neuhaus, G. and Schweiger, H.G. (1986) Expression of foreign genes in a higher plant cell after electrofusion-mediated cell reconstitution of a microinjected karyoplast and a cytoplast. *Eur. J. Cell. Biol.* **42**, 236–8.

Stefanov, I., Sándor, F., Bögre, L., Pauk, J., Fehér, A. and Dutis, D. (1994) Differential activity of the mannopine synthase and the CaMV 35S promoters during development of transgenic rapeseed plants. *Plant. Sci.* **95**, 175–86.

Velten, J. and Schell, J. (1985) Selection-expression plasmid vectors for use in genetic transformation of higher plants. *Nucl. Acid Res.* **13**, 6981–98.

Waldron, C., Murphy, E.B., Roberts, J.L., Gustafson, G.D., Armour, S.L. and Malcolm, S.K. (1985) Resistance to hygromycin B. *Plant Mol. Biol.* **5**, 103–8.

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Research Article

Evaluation of Various Sunflower (*Helianthus annuus* L.) Genotypes for *Agrobacterium tumefaciens*-mediated Gene Transfer

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Abstract: In this work, 10 sunflower (*Helianthus annuus* L.) genotypes were evaluated for their suitability for *Agrobacterium tumefaciens*-mediated gene transfer based on detection of a reported gene expression (GUS). A number of factors including the type of shoot-tip explant (split vs. intact), bacterial strain/binary vector combinations and wounding intact shoot-tips by particle bombardment were examined. Genotypes showed significant differences in their transformation efficiencies ranging from 0.0 to 82.7% GUS positive explants, hybrid genotypes being more responsive to *Agrobacterium* infection than inbred lines. Use of split shoot-tip explants did not increase the transformation efficiency over intact explants but the AGL-1/pKIWI strain/vector combination was more effective than the LBA4404/pTOK233 combination. Wounding explants by particle bombardment prior to inoculations with *Agrobacterium* had no positive effect on transformation.

Key Words: Sunflower, gene transfer, *Agrobacterium tumefaciens*, genotypes.

Çeşitli Ayçiçeği (*Helianthus annuus* L.) Genotiplerinin *Agrobacterium tumefaciens* Aracılığıyla Gen Transferi Açısından Değerlendirilmesi

Özet: Bu çalışmada, 10 ayçiçeği (*Helianthus annuus* L.) genotipinin, reporter bir genin (GUS) ekspresyonunun belirlenmesine dayanan *Agdobacterium tumefaciens* aracılığı ile gen transferine uygunluğu değerlendirilmiştir. Sürgün-ucu eksplant tipi (bölünmüş/split ve bölünmemiş/intact), bakteri suşu/binary vektör kombinasyonu ve bölünmemiş sürgün-ucu eksplantlarının partikülbombardımanı ile yaralanması gibi faktörler incelenmiştir. Transformasyon etkinligi bakımından genotipler arasında önemli farklılıklar gözlenmiş, ortalama %GUS pozitif eksplant oranları %0.0 ile %82.7 arasında değişmiştir. Hibrit genotipler, kendilenmiş hatlara göre *Agrobacterium* enfeksiyonuna karşı daha duyarlı olmuşlardır. Bölünmemiş (intact) eksplantlarla karşılaştırıldığında, bölünmüş (split) sürgün-ucu explantlarının kullanılması, transformasyon etkinliğini artırmazken AGL-1/pKIWI suş/vektör kombinasyonunun, LBA4404/pTOK233 kombinasyonuna göre daha etkili olduğu gözlenmiştir. Eksplantların, *Agrobacterium* ile inoküle edilmeden önce partikül bombardımanı ile yaralanmasının ise transformasyon üzerine herhangi bir olumlu etkisi görülmemiştir.

Anahtar Sözcükler: Ayçiçeği, gen transferi, *Agrobacterium tumefaciens*, genotipler.

Introduction

Increased disease resistance and oil content have been the main goals for the improvement of sunflower (*Helianthus annuus* L.) and achievement of these has been mainly restricted to conventional breeding methods. Because natural variations is a limiting factor in sunflower breeding, successful application of gene transfer techniques for the improvement of technological and agricultural qualities of sunflower varieties would be of great value. Development of a reliable gene transfer system, however, requires optimization of a number of variables. First, an efficient plant regeneration system from the target cells following transformation must be developed. There are several reports describing plant regeneration from different explants of sunflower including immature embryos (1, 2), cotyledons (3, 4) and thin cell layers from hypocotyls (5) but none of these systems has been conclusively shown to be applicable for transformation studies. Furthermore, the efficiency of plant regeneration has been hampered by the genotypic variation (6, 7).

A number of other factors affecting *Agrobacterium tumefaciens*-mediated transformation of sunflower have been studied. These included the physiological age of the explants (8), wounding explants prior to inoculations (9-11), *Agrobacterium* strain/vector combinations (9), co-cultivation period (8), hormonal composition of culture medium (12), and the type and the concentration of the

selection agents (13, 14) as well as the different gene transfer methods (15, 16). Use of shoot-tip meristems appears to be the only efficient method currently applied for genetic transformation of sunflower genotypes (12, 16, 17). This is simply because of the ease of regeneration of plants from the meristematic tissue in which shoot and leaf primordia already exist. Wounding of the shoot-tip meristems by microprojectiles (9, 10) or glass-beads (11) was also reported to increase the transformation efficiency. A more recent work, however, demonstrated that wounding immature zygotic embryos by microprojectile bombardment prior to bacterial inoculation had no effect on the transformation frequency of sunflower inbred lines (12). Although it has not been extensively studied, genotype dependency also appears to be a factor affecting the success of genetic transformation studies in sunflower (10). Therefore, the determination of the most responsive sunflower genotypes may be necessary for the establishment of an efficient gene transfer system.

In an effort to faciliate the transfer of genes encoding novel antimicrobial peptides into sunflower, we aimed to identify material which was most responsive to *Agrobacterium* infection. Using a subset of diverse sunflower genotypes (18), we examined the effects of the type of shoot-tip explant (split vs. intact), *Agrobacterium* strain/binary vector combinations and wounding of intact shoot-tips by particle bombardment on transformation efficiency.

Materials and Methods

Plant Material and Explant Preparation

Four commercial hybrids (Hysun 25, Hysun 36, Hysun 45 and Hyoleic 31) and six public inbred lines (HA 89, HA 341, RHA 271, DL 9542, DL 9546 and DL 9548) were used. Before sterilization, seeds were rinsed twice with sterile distilled water and then soaked in sterile distilled water for 2 h at room temperature. Seeds were washed in 70% ethanol for 2 min, dehulled and then rinsed in 70% ethanol for another 2 min followed by surface sterilisation in 2.5% sodium hypochlorite for 10 min under vacuum infiltration. Seeds were then rinsed again in 70% ethanol for 2 another min and finally rinsed in sterile distilled water 3-4 times followed by 5-6 hours imbibition in sterile distilled water at room temperature. Seed coats were removed and the seeds were cultured on MS-A medium, which contained full-strength MS salts and vitamins (19), 0.1 mg/l BAP (benzyladenine), 500 mg/l casamino acid (Difco), 30 g/l sucrose, 8 g/l bacto agar (Difco) at pH 5.7. After two days incubation in dark at 28°C, the cotyledons and radicles were removed and the explants were transferred back to the same medium for another two days in dark at 28°C. At the end of the 4 day's incubation (Figure 1A), the first two levels were removed to encourage the development of younger leaf primordia, and the remaining parts of the cotyledons and hypocotyls were trimmed down. Immediately after explant preparation, half of the explants were wounded by bombarding once with a particle inflow gun (20) using 5 µl of 0.5-1.2 µm size sterile tungsten particles (100 mg/ml) at 16 cm distance at 70 psi He pressure before bacterial inoculation and the other half of the explants were inoculated without wounding. Immediately after bombardment, one half of both the wounded and non-wounded explants were inoculated with the LBA4404/pTOK233 and the other half with the AGL-1/pKIWI strain/plasmid combinations. Also, to compare split explants with intact shoot-tips, split explants were prepared by cutting intact shoot-tips into two longitudinal halves before inoculations with LBA4404/pTOK233 or AGL-1/pKIWI combination.

Bacterial Strains/Binary Vectors and Co-cultivation

Two binary vectors, pTOK233 (21) kindly provided by T. Komari (Japan Tobacco Co., Japan) and pKIWI (22) by R. Gardner (University of Auckland, New Zealand), were used in combination with *A. tumefaciens* strains LBA4404 (23) and AGL-1 (24), respectively. The T-DNA region of both pTOK233 and pKIWI contained the *uid*A gene encoding GUS (β-glucuronidase), driven by 35S promoter of cauliflower mosaic virus. Presence of an intron in the *uid*A coding sequence in pTOK233 and the lack of ribosome binding site required by bacterial expression in pKIWI ensured that GUS expression could only be detected upon transfer of T-DNA into plant cells.

Overnight cultures of LBA4404/pTOK233 and AGL-1/pKIWI (OD_{600}=1.65) were grown in liquid YEP medium (25) with 50 mg/l kanamycin, 50 mg/l rifampicin and 200 µM acetosyringone by shaking in dark at 28°C at 180 rpm. The cultures were centrifuged at 4000 g for 10 min at 25°C and then resuspended in 10 mM $MgSO_4$, and used in co-cultivation. Wounded and non-wounded explants were immersed in bacterial suspension (diluted 10 times) for 45 mins and co-cultivated on MS-A medium for 3 days under 16/8 hours of light/dark regime at 25/20°C day/night temperatures. Following co-cultivation, explants were washed once in full-strength hormone-free MS liquid medium containing 30 g/l sucrose at pH 5.7 and then transferred to MS-B medium, which contained full-strength MS salts and vitamins, 0.5 mg/l BAP, 0.25 mg/l IAA (indole acetic acid), 0.1 mg/l

GA3 (giberellic acid), 500 mg/l cefotaxime, 50 mg/l kanamycin and 200 µM acetosyringone.

GUS Assay and Data Evaluation

After 5 days' incubation on MS-B medium, the explants were stained for GUS activity by immersing and vacuum infiltrating in GUS staining solution for 15 min and incubated overnight in dark at 37°C. The GUS staining solution was modified from Kosigo et al. (26) and contained 0.1% Triton x-100, 50 mM Na_2PO_4 at pH 7.0, 10 mM EDTA, 2 mM X-Gluc and 10% methanol. Chlorophyll from the stained tissue was removed by 70% ethanol.

Because the size of the isolated transformed tissue varied geratly, ranging from a very tiny sector consisting of a small group of cells to a large sector or a complete coverage of the leaf or hypocotyl, we decided to express the scoring in three different ways; i) mean number of GUS positive sectors per explant, ii) mean % of GUS positive area per explant and iii) % of explants showing GUS activity. In each treatment, 45-60 explants were used, and all of the explants used were taken into account when calculating the means. SE (standard error of the mean) values were also calculated for the parameters (i) and (ii).

Results and Discussion

Comparison of Split and Intact Shoot-tip Explants

Previously, it was shown that the split shoot-tip explants were more effective in the production of transgenic sunflower plants (10). We, therefore, first compared the split and intact explants of Hysun 36 following inoculations with either LBA4404/pTOK233 or AGL-1/pKIWI combination. Our results suggested that transformation efficiency was not changed with the use of split explants (Table 1). This was consistent with the recent work of Burrus et al. (12) who found that split and intact meristem explants produced comparable transformation rates in public and experimental inbred lines. We also observed that shoot development was considerably reduced as a result of splitting and most of the split explants produced only callus which failed to regenerate into shoots. This might be due to the damage to the shoot-tip meristems caused by cutting which then appeared to provoke necrosis after co-cultivation with *Agrobacterium*, as also reported by others (16). This finding is, however, contrary to the previous work of Knittel et al. (10) who reported that a longitudinal section through the apical meristem favoured multiple shoot induction and subsequent gene transfer into the cut region. Our results suggested that the type of shoot-tip explant (split or intact) may not be very important while the existence of rapidly dividing cells in the meristem, which are potential targets for the *Agrobacterium*, is probably a more critical factor. However, it may be important that other tissues surrounding the meristematic region be removed to facilitate full exposure of the meristematic cells to to bacteria.

Comparison of Different Genotypes

Ten sunflower genotypes, which contained a reasonable diversity based on genetic similarity values previously assessed by AFLP analysis (18), were compared for their transformation efficiency. The method we used to evaluate different genotypes assessed the transformation efficiency based on whether the *gus* reporter gene had been successfully transferred into young explants. Although successful recovery of fertile transgenic plants at reasonable frequencies should be a key criterion for evaluating the effectiveness of a gene transfer protocol, our method represented an easy and convenient way of evaluating the responsiveness of each genotype to the transformation conditions employed.

Our results showed that genotypes differed significantly in terms of all three parameters assessed (Table 2). Hysun 45 was the most responsive genotype with the highest number of GUS positive sectors and the largest % of transformed area per explant when compared in terms of the mean of the four treatments. However, when individual treatments were compared, the responses were varying among cvs. Hysun 25, Hysun 36

Table 1. Comparison of split and intact shoot-tip explants from cv. Hysun 36 following inoculation with LBA4404/pTOK233 or AGL-1/pKIWI. Values are the mean±SE (45-60 explants per treatment).

Parameters	Treatments			
	Split/LBA4404	Intact/LBA4404	Split/AGL-1	Intact/AGL-1
Mean number of GUS positive sectors per explant	3.6±0.18	3.5±0.26	3.4±0.21	3.6±0.22
Mean % of GUS positive area per explant	2.4±0.22	2.0±0.13	2.7±0.17	3.8±0.31
% of GUS positive explants	72.1	62.8	68.6	78.0

Table 2. Comparison of transformation efficiencies of 10 sunflower genotypes after inoculations with LBA4404/pTOK233 (L) or AGL-1/pKIWI (A) with (+) or without (-) wounding with a particle inflow gun. Values are the mean±SE (45-60 explants per treatment).

Genotypes	Mean number of GUS positive sectors per explant					Mean % of GUS positive area per explant					% of GUS positive explants				
	L/+	L/-	A/+	A/-	Mean	L/+	L/-	A/+	A/-	Mean	L/+	L/-	A/+	A/-	Mean
Hysun 25	1.7±0.2	2.5±0.3	3.8±0.6	3.1±0.4	2.8±0.4	5.7±0.4	2.0±0.4	1.9±0.3	3.1±0.2	3.2±0.3	77.8	87.4	62.5	77.2	76.2
Hysun 36	5.2±0.7	3.8±0.4	4.8±0.5	1.8±0.3	3.9±0.5	1.5±0.1	1.6±0.1	4.4±0.4	2.6±0.3	2.5±0.2	59.3	81.2	83.4	79.6	75.9
Hysun 45	4.9±0.5	3.3±0.3	6.2±0.7	6.7±1.0	5.3±0.6	5.4±0.8	2.6±0.7	2.7±0.4	14.2±2.1	6.2±1.0	83.4	55.4	91.6	100	82.7
Hyoleic 31	2.4±0.3	2.9±0.5	3.8±0.2	4.9±0.5	3.5±0.4	1.2±0.2	1.9±0.4	1.4±0.4	4.0±0.7	2.1±0.4	54.5	90.0	72.0	73.0	72.4
HA 89	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.1	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.1	9.1	0.00	0.00	0.00	2.3
Ha 341	1.0±0.2	1.1±0.2	0.8±0.1	1.4±0.3	1.1±0.2	0.6±0.1	1.6±0.3	0.6±0.2	1.0±0.3	1.0±0.3	50.0	42.8	25.0	57.0	43.7
RHA 271	0.9±0.3	2.3±0.5	0.5±0.2	0.0±0.0	1.0±0.3	1.2±0.1	3.4±0.3	0.3±0.1	0.0±0.0	1.2±0.1	38.5	75.0	23.0	0.00	34.2
DL 9542	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.00	0.00	0.00	0.00	0.00
DL 9546	0.6±0.2	0.7±0.2	1.2±0.3	2.2±0.6	1.2±0.3	1.0±0.1	0.5±0.1	0.9±0.1	1.3±0.2	0.9±0.1	37.5	28.6	54.8	47.0	42.0
DL 9548	1.5±0.3	2.4±0.4	6.6±0.8	2.6±0.4	3.3±0.5	1.3±0.2	0.9±0.1	1.8±0.5	2.2±0.3	1.6±0.3	46.4	56.2	83.4	55.0	60.3

and Hysun 45. The hybrid genotypes, together with DL 9548 inbred line, were distincively better than the rest of the genotypes. The percentage of GUS positive explants in our hybrid genotypes was similar to those found by Knittel et al. (10) for the shoot-tip meristem explants. Seeds from hybrid genotypes germinated more vigorously than those from other genotypes and this suggests that there may be a relationship between the vigorous growth and the higher transformation efficiencies of these genotypes. These results indicated that the capacity of individual genotypes for transformation is determined in large part by their genotypes. Therefore, testing the capability of a given genotype or breeding material of sunglower for *Agrobacterium* infection should be a prerequisite when optimizing a transformation system.

After co-cultivation with *Agrobacterium*, GUS expressing sectors of varying sizes were found on leaves, hypocotyls and meristematic region (Figure 1B-I). GUS positive sectors either appeared at the distal end of the lamina only (Figure 1B) or were scattered all over the leaf lamina (Figure 1C), and in some cases, covered the whole stem (Figure 1H) or leaf (Figure 1I). Most of the small GUS positive sectors could be related to the infection of the particle-wounded cells by *Agrobacterium*. The transformed cells located at the leaf tip or in small clusters of cells in the lamina appear to be origination from the cells in the leaf primordium which were present at the time of bombardment and/or inoculation. Occasionally, GUS staining was associated with the vascular tissue as sectors along the leaf midrib (Figure 1G), meristematic region (Figure 1D, E) or cell lineages from the petiole base to the lamina tip (Figure 1F). The sectors running the length of the leaf could be interpreted as arising from transformation of a meristematic cell proior to the initiation of the leaf primordium. The expression was excepter to be stable as GUS positive areas on the explants were observed even 20-30 days after co-cultivation. It is possible that such transformation events will give rise to plants which are usually chimeric for the transferred gene. However, fully transformed individuals can be isolated in the next generation from the progeny of chimeric plants. This can be done by germinating the seeds collected from chimeric plants (primary transformants) on a medium containing kenemycin resistant indivuduals can later be confirmed by GUS assays and other molecular analyses (i.e. southern blot analysis).

Effect of Bacterial Strain/Binary Vector Combination

We also compared the effect of two different *Agrobacterium* strain/binary plasmid vector combinations on the transformation efficiency. The LBA4404/pTOK233 has been successful in *Agrobacterium*-mediated transformation of monocotyledons such as rice (21) and maize (27). The AGL-1 strain is known to be a 'super virulent' on a number of plant species (24). Although the two bacterial strains we used are not directly comparable since they carry different binary vectors, our results suggested that the AGL-1/pKIWI was more effective than the LBA4404/pTOK233, producing approximately 35% more GUS positive sectors (Figure 2A) and 30% larger GUS positive area (Figure 2B). Both combination, however, produced similar proportions of GUS positive shoots (Figure 1C). The slightly better transformation efficiency of AGL-1/pKIWI might be due to higher virulence of this strain on sunflower as the other nopaline type *A. tumefaciens* strains, such as EHA101, were also

Figure 1. a) A typical sunflower explant after the removal of cotyledons and radicle (2 days), and before removal of first two leaves (4 days). Inoculation with or without particle bombardment was performed immediately before leaves were removed b) GUS expression located mainly at the distal end or c) scattered all over the lamina. d, e) Explants showing GUS expression on the meristematic region as well as on the leaves and hypocotyl. f) Transformed cell lineages running from the hypocotyl base to the lamina tip or g) associated with the vascular tissue as sectors along the leaf midrib. h) GUS expression on the hypocotyl and i) leaves.

reported to be more effective for sunflower transformation (9).

Effect of Wounding by Particle Bombartment

Wounding the meristematic region by particle bombardment or treatment with glass-beads has been reported to be critical for the recovery of transgenic shoots in sunflower (9-11). We, therefore, compared the transformation efficiencies of wounded and non-wounded intact shoot-tip explants of the same 10 genotypes using a single He pressure at 70 psi. Our results showed that the wounded explants had slightly higher numbers of GUS positive sectors than the non-wounded explants, 2.29 sectors per explant compared to 2.10 (Figure 2A). However, when mean % GUS positive area per explart is cmopared, non-wounded explants had approximately 35% more GUS positive tissue than the wounded explants (Figure 2B). In a later experiment, we also compared the effects of a range of bombardment presssures (0, 40, 70 and 100 psi) using two of the most responsive genotypes (Hysun 45 and Hysun 36) and observed that increased bombardment pressures reduced both the number of GUS positive sectors and % of transformed area irrespective of the bacterial strain/plasmid combinations (data not given). These findings are different from the results of Bidney *et al.* (9)

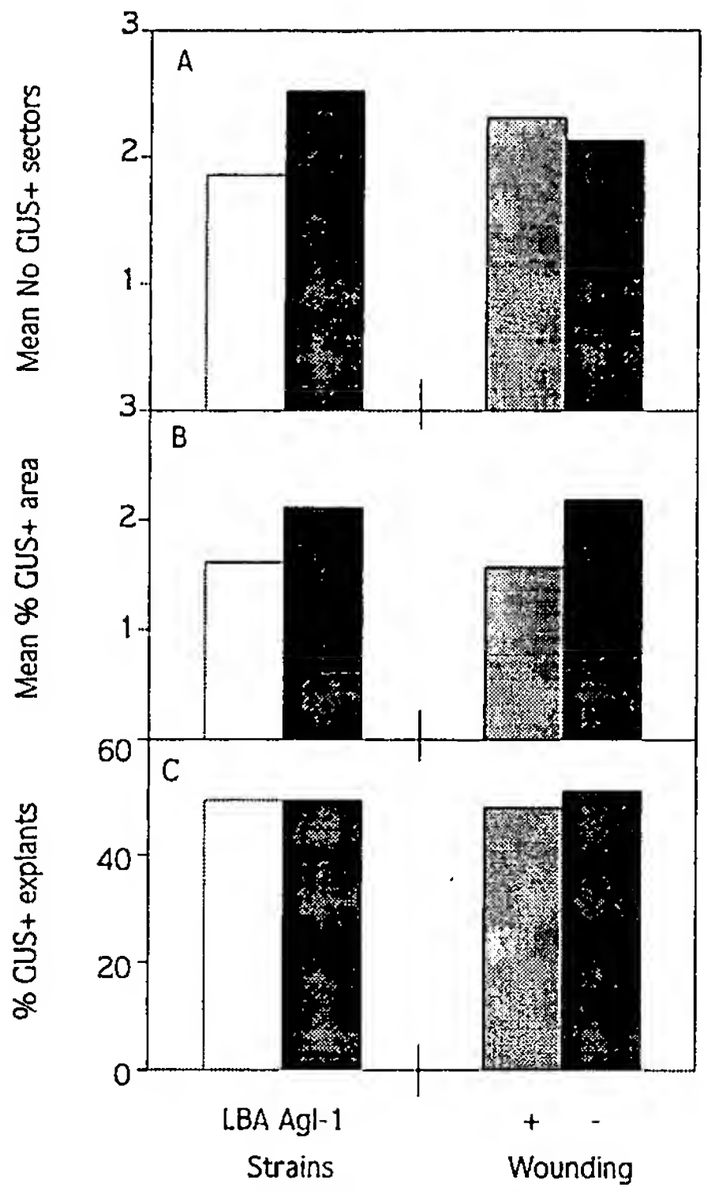

Figure 2. Comparison of the LBA4404/pTOK233 and AGL-1/pKIWI strain/binary vector combinations, and the wounded and non-wounded shoot-tip explants in terms of the mean number of GUS positive sectors per explant (A), mean % of GUS positive area per explant (B) and mean % of GUS positive explants (C). The results are the means of 10 genotypes.

and Knittel *et al.* (10) who found a marked increase in transformation frequency after wounding sunflower shoot-tip meristems. In the latter work, 41% of the explants showed GUS activity when wounded whereas only 11% showed activity in the non-wounded explants (10). In our experiments, both wounding and non-wounding treatments resulted in the production of similar proportions of GUS positive explants, 47.7 and 50.3% (Figure 2C). This is consistent with a recent work by Burrus et al. (12) in which wounding by bombardment before *Agrobacterium* inoculation did not increase the transformation efficiency.

Conclusions

Our results suggest that the proper selectin of genotype and the *Agrobacterium* strain/vector combination may be critical in sunflower transformation. Wounding by particle bombardment or the use of split explants did not have any positive effect on the overall efficiency of transformation in those experiments. However, whether these factors will be important in obtaining stably transformed sunflower plants still awaits further investigation.

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References

1. Finer JJ, direct somatic embryogenesis and plantlet regeneration from immature embryos of hybrid sunflower (Helianthus annuus) on a high sucrose-containing medium. Plant Cell Rep., 6: 373-374 (1987).
2. Freyssinet M, Freyssinet G. Fertile plant regeneration from sunflowir (helianthus annuus) immature embryo. Plant Sci., 56: 177-181 (1988).
3. Ceriani MF, Hopp HE, Hahne G, Escandon AS, Cotyledons: An expland for routien regeneration of sunflower plants. plant Cell physiol., 33: 157-164 (1992).
4. Fiore MC, Trabace T, Sunseri F. High frequency of plant regeneration in sunflower from cotyledons via somatic embryogenesis. Plant Cell Rep., 16: 295-298 (1997).
5. Pelissien B, Bouchefra O, Pepin R, Freyssinet G. Production of isolated somatic embryos from sunflower thin cell layers. Plant Cell Rep., 9: 47-50 (1990).
6. Sarrrafi A, Bolandi AR, Serieys H, Berville A, Alibert G, Analysis of cotyledon culture to measure genetic variability for organogenesis parameters in sunflower (Helianthus annuus L.). Plant Sci., 121: 213-219 (1996).
7. Deglene L, Lesignes P, Alibert G, Sarrafi A, Genetic control of organogenesis in cotyledons of sunflower (Helianthus annuus). Plant Cell Tiss. Org. Cult., 48: 127-130 (1997).
8. Schrammeijer B, Sijmons PC, Van Den Elzen PJM, Hoekema A, Meristem transformation of sunflower via Agrobacterium. Plant Cell Rep., 9: 55-60 (1990).

9. Bidney D, Scelonge C, Martich J, Burrus M, Sims L, Huffman G, Microprojectile bombardment of plant tissues increases transformation frequency by Agrobacterium tumefaciens. Plant Mol. Biol., 18: 301-313 (1992).

10. Knittel N, Gruber V, Hahne G, Lenee P, transformation of sunflower (Helianthus annuus L.): A reliable protocol. Plant Cell Rep., 14: 81-86 (1994).

11. Grayburn WS, Vick BA, Transformation of sunflower (helianthus annuus L.) following wounding with glass beads. Plant Cell Rep., 14: 285-289 (1995).

12. Burrus M, Molinier J, Himber C, Hunold R, Bronner R, Rousselin P, Hahne G, Agrobacterium-mediated transformation of sunflower (Helianthus annuus) shoot apices: transformation patterns. Mol. Breed., 2: 329-338 (1996).

13. Escandon AS, Hahne G, Genotype and composition of culture medium are factors important in the selection for transformed sunflower (Helianthus annuus) callus. Physiol. Plant., 81: 367-376 (1991).

14. Pugliesi C, Biasini MG, Fambrini M, Baroncelli S, Genetic transformation by Agrobacterium tumefaciens in the interspecific hybrid Helianthus annuus x Helianthus tuberosus. Plants Sci., 93: 105-115 (1993).

15. Moyne AL, Tagu D, Thor V, Bergounioux C, Freyssinet G, Gadal P, Transformed calli obtained by direct gene transfer into sunflower protoplasts. Plant Cell Rep., 8: 97-100 (1989).

16. Laparra H, Burrus M, Hunold R, Damm B, Bravo-Angel AM, Bronner R, Hahne G, Expression of foreign genes in sunflower (Helianthus annuus L.): Evaluation of three gene transfer methods. Euppytica, 85: 63-74 (1995).

17. Malone-Schoneberg J, Scelonge CJ, Burrus M, Bidney DL, Stable transformetion of sunflower using Agrobacterium and split embryonic axis explants. Plant Sci., 103: 199-207 (1994).

18. Hongtrakul V, Huestis GM, Knapp SJ, Amplified fragment length polymorphisms as a tool for DNA fingerprinting sunflower germplasm: genetic diversity among oilseed inbred lines. Theor. Appl. Gen., 95: 400-407 (1997).

19. Murashige T, Skoog F, A revised medium for rapid growth and bioassays with tobacco tissu cultures. Physiol. Plant., 15: 473-497 (1962).

20. Finer JJ, Vain P, Jones MW, McMullen MD, Development of the particle inflow gun for DNA delivery to plant cells. Plant Cell Rep., 11: 323-328 (1992).

21. Hiei Y, Ohta S, Komari T, Kumashiro T, Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacteriam and sequence analysis of the boundries of the T-DNA. The Plant J., 6: 271-282 (1994).

22. Janssen BJ, Gardner RC, Localized transiet expression of GUS in leaf discs following coclutivation with Agrobacterium. Plant Mol. Biol., 14: 61-72 (1989).

23. Hoekama A, Hirsch PR, Hoyykaas PJJ, Schilperoort RA, A binary plant vector strategy based separation of vir- and T-region of the Agrobacterium tumefaciens Ti plasmid. Nature, 303: 179-180 (1983).

24. Lazo GR, Stein PA, Ludwig RA, A DNA transformation-competent Arabidopsis genomic library in Agrobacterium. Bio/Tech., 9: 963-967 (1991).

25. An G, Ebert PR, Mitra A, Ha SB, Binary vectors. In: Gelvin SB, Schilperoort RA, Verma DPS (eds.) Plant Molecular Biology Manual. Kluwer Academic Publishers, Dordrecht, The Netherlands. pp. A3/1-19 (1988).

26. Kosigo S, Ohashi Y, Nakajima K, Arai Y, An improved assay for β-glucuronidase in transformed cells: Methanol almost completely suppresses a putative endogenous β-glucuronidase activity. Plant Sci. 70: 133-140 (1990).

27. Ishida Y, Saito H, Ohta S, Hiei Y, komari T, Kumashiro T, High efficiency transformation of maize (Zea mays L.) mediated by Agrobacterium tumefaciens. Nature Biotech., 14: 745-750 (1996).

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Effects of tomato cultivar, leaf age, and bacterial strain on transformation by *Agrobacterium tumefaciens*

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Abstract

Differences in transformation of the tomato cultivar (Ohio 7870, Roma, UCD82b) by wild-type *Agrobacterium* strains (A6, A66, A281) were identified in a leaf disk assay system. Transformation was expressed as the percentage of explants producing callus on hormone-free medium and was confirmed by opine production. Ohio 7870 and Roma were more readily transformed than UCD82b by all three strains of *A. tumefaciens*. Cotyledons and older true leaves of all three cultivars were more readily transformed than younger leaves. Transformation was biphasic over the bacterial concentrations tested (2×10^3–7×10^9 colony forming units ml^{-1}; cfu ml^{-1}) for all cultivars and leaf ages, and was greatest at 5×10^8 cfu ml^{-1}. Transformation decreased significantly at levels less than 2×10^7 cfu ml^{-1} and slightly at concentrations higher than 5×10^8 cfu ml^{-1}. UCD82b tissue was more necrotic than Ohio 7870 or Roma after incubation with bacteria, which may account for reduced transformation of this cultivar.

Introduction

Leaf disks [1–4] and stem segments [5,6] are two in vitro *Agrobacterium*-based transformation systems reported for tomato. Varying rates of transformation were reported (3 percent to 70 percent) in these studies using disarmed bacteria and subsequent regeneration of kanamycin-resistant plants as a measure of transformation. Although regenerated transformed plants was the desired goal, the final number of regenerated plants was dependent on expression of kanamycin resistance in addition to transformation. Therefore, variation in percent transformation was probably due to several factors.

The sequence of interactions between plant and *A. tumefaciens* cells and the regulatory controls exerted by each organism during transformation are not fully understood. There have been numerous plant tissue studies with many species which document transformation by *A. tumefaciens* [1–6,12–15]. However, there has been little research done to study the effects of specific plant host factors such as plant cultivar, host plant response to various bacterial strains and host tissue age on transformation by *A. tumefaciens*. The aim of the work reported here was to study transformation differences among tomato cultivars by evaluating the effect of bacterial inoculum density, bacterial strain, and leaf age on transformation.

Materials and methods

Plant tissue

Tomato cultivars

Three processing tomato cultivars were used: Ohio 7870, UCD82b, and Roma. Seeds were obtained from Dr. S. Berry, OARDC, Wooster, Ohio (Ohio

7870 and Roma), and Dr. J. Hewitt, Veg. Crops Dept., U. of California, Davis (UCD82b).

Explant preparations

Seeds were sterilized for 20 minutes in 20% (v/v) Clorox (1% Na-hypochlorite) and then germinated aseptically in glass jars containing moist filter paper. Seven-day-old cotyledons were excised near the axis. Leaf pieces (approximately 1 cm^2) were cut from the three terminal leaflets of leaves of 6-week-old greenhouse-grown plants (plants had 6 to 7 leaves total). Leaf 1 (most distal from the apex) was the oldest leaf (of normal morphology) and leaf 5 (most proximal to the apex) was the youngest. Each leaf piece contained a part of the midrib. Leaf tissue was sterilized 10 minutes in 10% (v/v) Clorox (0.5% Na-hypochlorite) plus 0.1% (v/v) Tween 20.

Transformation procedures

A. tumefaciens culture

Three wild-type strains of *A. tumefaciens* known to cause transformation in tomatoes were used (A281, A6, A66). Strain A281 (pTiBo542) was obtained from Dr G. An, Washington State University. Strains A6 (pTiA6) and A66 (pTiA66) were obtained from Dr Andy Binns, University of Pennsylvania. Overnight cultures were grown in tryptone yeast extract glucose medium and diluted to specified concentrations with MSO medium ([8]; 1/10 salts plus full organics).

Inoculation

Explants were dipped into the bacterial suspension, blotted dry, and plated onto agar-solidified MSO medium abaxial side up. Explants were incubated for 2 days at 24°C under a 16 h light:8 h dark photoperiod from cool white fluorescent light at 85 $\mu mols\,m^{-2}sec^{-1}$. Bacteria were removed by dipping the tissue into MSO + 500 $\mu g\,ml^{-1}$ Geopen (filter-sterilized carbenicillin) briefly and then plating onto full strength MS medium containing 3% sucrose and 0.7% agar. Transformation was demonstrated by callus (tumor) growth on the hormone-free medium and verified by production of agropine (strain A281) or agropine and octopine (strains A6, A66) by paper electrophoresis [9] after 3 weeks of culture. For all experiments, controls consisted of non-inoculated plant tissue placed on hormone-free MS medium to confirm no growth of callus tissue without *A. tumefaciens* and on MS medium + 2 $mg\,l^{-1}$ zeatin to confirm the potential for organogenesis. Controls were dipped into bacterial culture medium filtrate.

Data analysis

Randomized complete block experiments were performed with at least two replicate plates containing 5 to 10 explants/plate. Experiments were replicated in time. Percentage data was transformed by the method of Anscombe [10] to provide a distribution with a variance constant over the entire range of proportions. Analysis of variance was performed and mean separation was inferred with Tukey's studentized range test. Statistical methods were those suggested for use in tissue culture experiments by Mize & Chun [11].

Results

After exposure to *A. tumefaciens* and plating on hormone-free medium, cotyledons showed tumor formation (Fig. 1). Controls, after exposure to bacterial culture medium filtrate, on hormone-free MS medium showed massive root proliferation and those on MS + zeatin showed callus and shoot proliferation. All types of tissue tested were transformed by *A. tumefaciens* although differences in transformation efficiency were seen among the bacterial concentrations, bacterial strains and leaf ages tested.

Effect of bacterial concentration on transformation

Transformation of cotyledons by strain A281 was biphasic regardless of tomato cultivar (Fig. 2). Transformation was low at 10 to 5 × 10^5 cfu ml^{-1}, increased significantly to its maximum at 5 × 10^8 cfu ml^{-1} and decreased at higher concentrations. There was a cubic relationship between the percent transformation and bacterial concentration ($P = 0.0122$). When compared to Ohio 7870 and Roma, a lower percentage of UCD82b cotyledons was transformed at all levels of *A. tumefaciens* but this was not significant ($P = 0.05$).

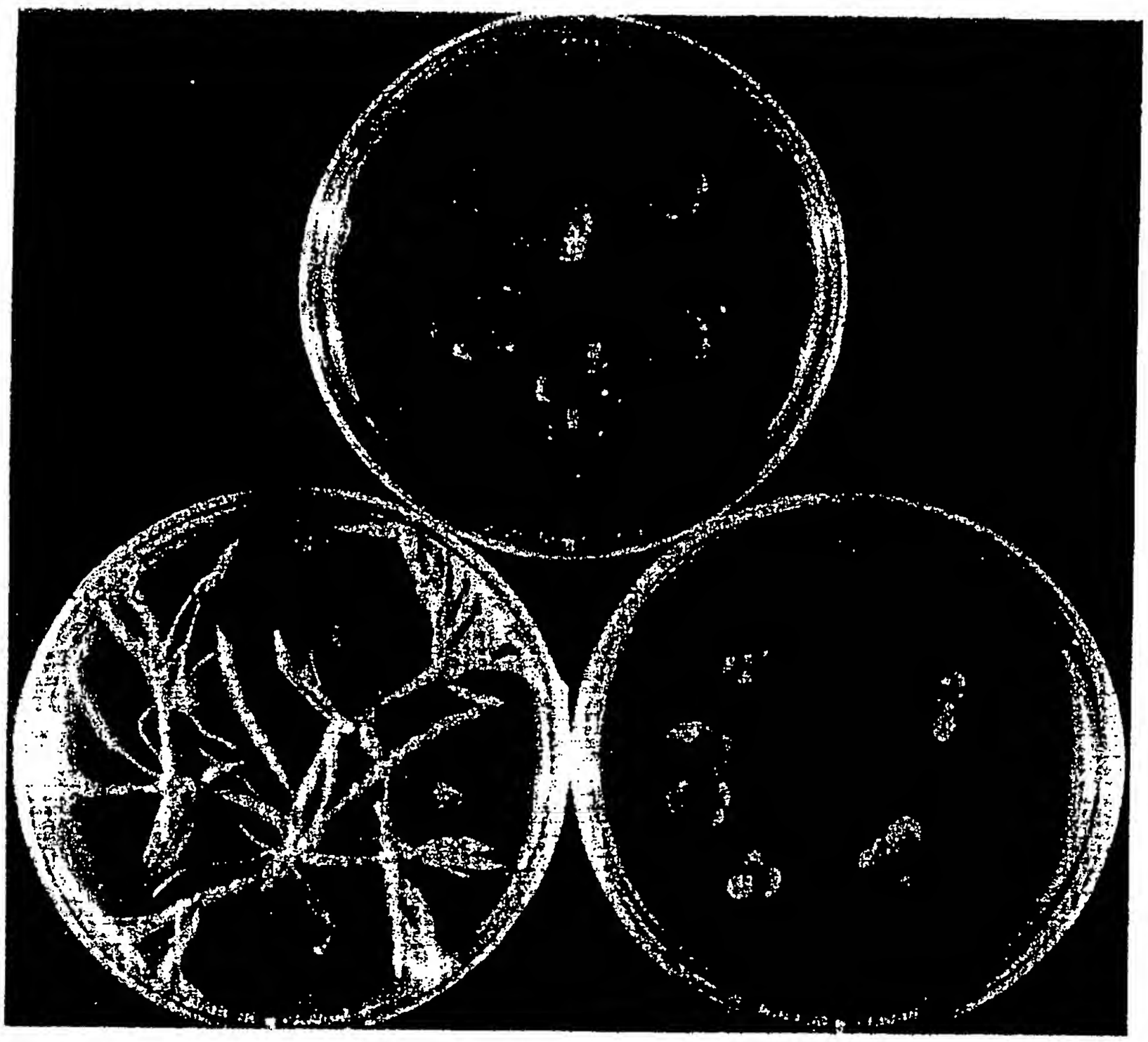

Fig. 1. Representative Ohio 7870 cotyledons after three weeks. After exposure to *A. tumefaciens* and plating on hormone-free MS medium, cotyledons showed tumor formation (bottom right). Those controls not exposed to *Agrobacterium* on hormone-free MS medium showed massive root proliferation (bottom left) and those on MS + zeatin showed callus and shoot proliferation (top).

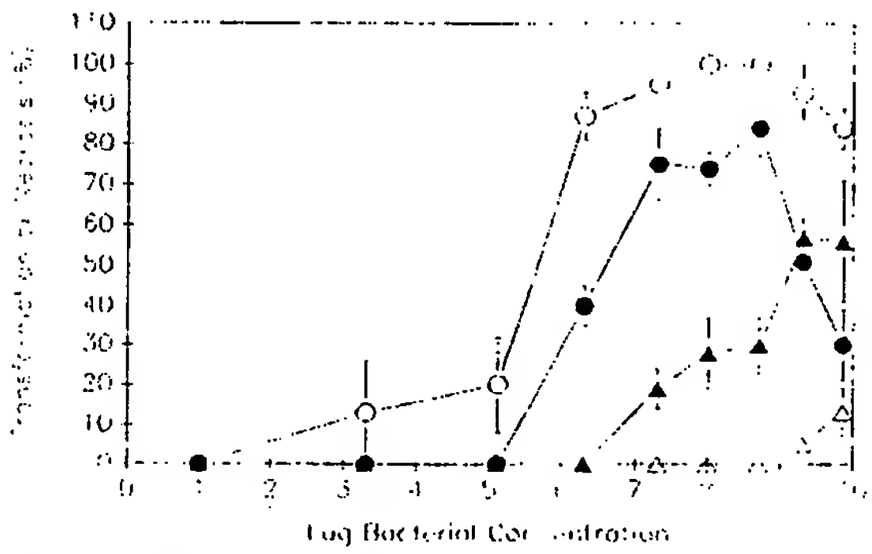

Fig. 2. The effect of *A. tumefaciens*, strain A281 concentration (colony forming units/ml) on percent transformation of cotyledons of cultivars Ohio 7870 (○), UCD82b (●), percent necrosis of Ohio 7879 (△), UCD82b (▲). Vertical bars represent the standard error. Data were combined from two experiments. Total number of explants/treatment was 50.

More necrosis of explants was seen at the highest concentrations of bacteria for all cultivars. There was a significant ($P = 0.0001$) linear relationship between the percentage of necrotic explants and bacterial concentration, and there was a significant ($P = 0.0001$) interaction of cultivar with bacterial concentration. UCD82b was more susceptible to necrosis than Ohio 7870 at higher concentrations of bacteria.

Generally, the number of tumors per cotyledon ranged between two and six. Bacterial concentrations of 1×10^8 or 5×10^8 cfu ml^{-1} resulted in the largest tumors for all cultivars, and Ohio 7870 tumors were larger than those on Roma or UCD82b (data not shown).

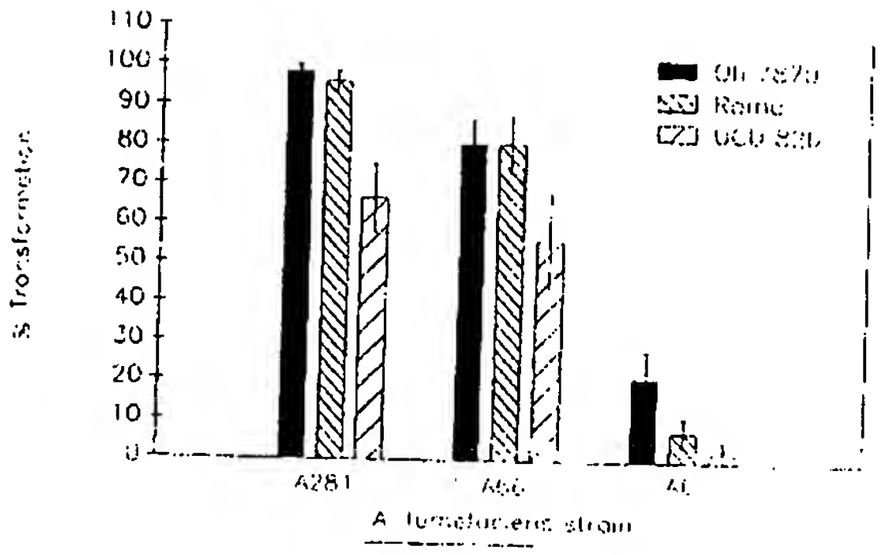

Fig. 3. The effect of bacterial strain on percent transformation of cotyledons of Ohio 7870, Roma, and UCD82b tomato cultivars by *A. tumefaciens*. Standard error bars are shown. Data were combined from two similar experiments. 2–4 × 10^8 colony forming units/ml bacteria were used. Total number of explants/treatment was 50.

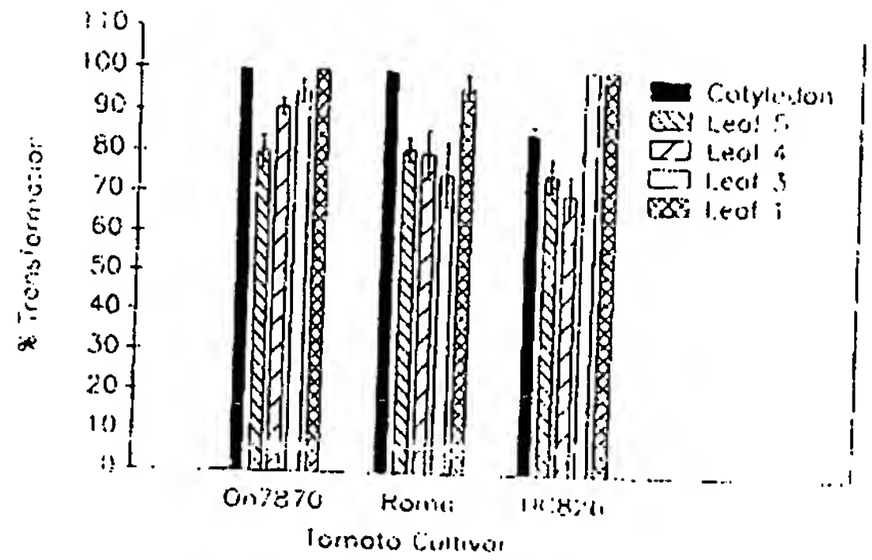

Fig. 4. The effect of leaf age on percent transformation of Ohio 7870, Roma, and UCD82b tomato cultivars *A. tumefaciens*, strain A281. Standard error bars are shown. Data were combined from two experiments with 2–5 × 10^8 colony forming units bacteria/ml. Total number of explants/treatment was 20. Leaf 1 was the oldest leaf; leaf 5 was the youngest leaf.

Effect of bacterial strain on transformation

Using three wild-type strains of *A. tumefaciens* (2–4 × 10^8 cfu ml^{-1}), there was a significant difference ($P = 0.0001$) in the transformation of the three tomato cultivars (Fig. 3). A higher percentage of Ohio 7870 and Roma cotyledons were transformed than UCD82b regardless of bacterial strain. There was also a significant difference ($P = 0.0001$) in percent transformation caused by different bacterial strains. Strain A6 resulted in far less tumor formation than either A281 or A66 and A66 resulted in less than A281.

A low concentration of strain A6 (3 × 10^6 cfu ml^{-1}) resulted in no transformation (data on transformation using different concentrations of A6 not shown). A high concentration (3 × 10^{10} cfu ml^{-1}) resulted in a lower percent transformation for Roma and UCD82b, and a lower or similar level of percent transformation for Ohio 7870 compared to a bacterial concentration of 5 × 10^8 cfu ml^{-1}. Strain A6 also caused greater amounts of necrosis at lower concentrations in UCD82b than in the other two cultivars and caused greater amounts of necrosis in all cultivars than the strains A281 and A66.

Effect of leaf age on transformation

After inoculation with strain A281, tomato leaves of all ages formed tumors although there was a significant difference ($P = 0.0001$) between the percent transformation of different age leaves (Fig. 4). Young leaves (Leaf 5) exhibited a lower percent transformation than old leaves (Leaf 1) in all three cultivars. Old leaves (Leaf 1) of all three cultivars were transformed at percentages comparable to cotyledons using strain A281. Compared to strain A281, strains A6 and A66 caused very low levels of transformation and more necrosis of leaves (data not shown). Ohio 7870 leaves, like cotyledons, were less susceptible to necrosis caused by higher concentrations of bacterial strain A281 than UCD82b (Table 1). Old leaves (Leaf 1) of both Ohio 7870 and UCD82b showed a higher percentage of transformation and less necrosis than young leaves (Leaf 5; Table 1). Roma was not tested.

Shoot proliferation after transformation with strains A66 or A281

Cotyledons of all three cultivars produced shoots after transformation with strain A66 or A281 (Table 2). Although individual shoots formed after transformation were not evaluated for opine content, callus from which these shoots were derived did produce opines (data not shown). More Ohio 7870 tumors produced shoots than Roma tumors and both produced more than UCD82b tumors. Similar number of shoot-producing tumors resulted from transformation of Ohio 7870 by the wild-

Table 1. Effect of *A. tumefaciens* strain A281 concentration, on the number of old and young leaves of Ohio 7870 and UCD82b transformed and the number exhibiting necrosis[1].

Bacterial conc. (cfu ml^{-1})[2]	Ohio 7870		UCD82b	
	Leaf 1[3]	Leaf 5	Leaf 1	Leaf 5
	Number transformed/Total number of explants			
8×10^5	6/6	3/8	9/9	2/7
8×10^8	6/6	7/8	8/8	1/8
1×10^{10}	6/6	6/7[4]	2/4[4]	3/6[4]
	Number necrotic/Total number of explants			
	3/18	15/23	12/21	19/21

[1] Data presented is from a representative experiment which was done twice.
[2] Bacterial concentration is expressed in colony forming units/ml (cfu ml^{-1}).
[3] Leaf 1 was the oldest leaf of normal morphology and Leaf 5 was the most proximal to the apex.
[4] Tumors were small.

type strain A281 and the shoot-inducing strain A66. Fewer shoot-producing tumors resulted from transformation of Roma or UCD82b by strain A66 than strain A281. Tumors incited by strains A281 or A66 on old leaves did not produce shoots. Shoot production by tumors incited by strain A281 on Ohio 7870 cotyledons was biphasic over the range of bacterial concentrations tested. The highest percentage of explants producing shoots occurred at 5×10^8 cfu ml^{-1} and the percentage of explants producing shoots was lower at lower or higher concentrations of bacteria.

Table 2. Effect of *A. tumefaciens* strain and concentration on percent of cotyledons forming shoots for three tomato cultivars.

Bacterial stain	Bacterial concentration (cfu ml^{-1})[1]	Percent of cotyledons forming shoots		
		Ohio 7870	Roma	UCD82b
A66	3×10^8	60 ± 9[2]	39 ± 11	25 ± 12
A281	2×10^6	7 ± 7	0 ± 0	0 ± 0
	2×10^7	31 ± 12	8 ± 4	0 ± 0
	1×10^8	66 ± 4	4 ± 4	2 ± 2
	5×10^8	68 ± 14	10 ± 4	6 ± 4
	2×10^9	25 ± 8	2 ± 2	0 ± 0
	7×10^9	17 ± 3	16 ± 7	0 ± 0

[1] Bacterial concentration is expressed in colony forming units/ml (cfu ml^{-1}).
[2] Data presented is the mean ± standard error. Data is combined from two experiments with 50 cotyledons/treatment (Strain A281), and 60 explants/treatment (Strain A66). Each treatment was replicated five times with 10–12 cotyledons/replicate.

Discussion

Transformation was judged by tumor formation in these experiments and confirmed by opine production. It was possible that transformation occurred without visible tumors and with little or no opine production, but this possibility was not tested.

Tomato cultivar affected transformation although the differences between the three cultivars examined in this study (Figs 2,4) were generally small. Ohio 7870 tissue was transformed more readily and was more resistant to necrosis than UCD82b as seen by a higher percentage of transformation for all three bacterial strains (Fig. 3) and greater transformation and less necrosis at all concentrations of bacteria for both cotyledons and leaves (Fig. 4, Table 1). Ohio 7870 cotyledons may have traits which allow a larger number of transformation events to occur as reflected by the higher percentage of explants transformed with Ohio 7870 (Figs 2,3). Ohio 7870 may also have different response compared to Roma and UCD82b for expression of the inserted *A. tumefaciens* genes (*onc* genes) from strains A66 and A281, as reflected by the greater percentage of shoot formation (Table 2). Larger tumors seen on Ohio 7870 after transformation by strain A281 could be caused either by factors which allow more transformation events to occur or by differing response to *onc* gene expression. Plant cultivar was also found to be an important factor in determining transformation frequencies in moth bean (*Vigna aconitifolia*) in which one cultivar was found to have an 85-fold higher transformation rate than another [12].

The optimal bacterial concentration found using strain A281 (1 to 5 × 10^8 cfu ml^{-1}) (Fig. 2) was similar to that found by Filatti et al. [3] using UCD82b cotyledons and an engineered derivative of strain LBA4404. A bacterial concentration of 1 to 5 × 10^8 cfu ml^{-1} resulted in the largest tumors (data not shown) and the greatest shoot formation (Table 2) for cotyledons of all three cultivars which may indicate a larger number of transformations or an optimal plant response to expression of the *onc*

genes. The same approximate bacterial concentration (3×10^8–3×10^9 cfu ml^{-1}) was also optimal for percent transformation of cotyledons of three cultivars by strain A6 (data not shown).

The necrotic response was greater in UCD82b than Ohio 7870 or Roma (Fig. 2, Table 1). This necrosis was probably due to a hypersensitive response of the tomato tissue. Necrotic effects resulting from a plant hypersensitive response to *A. tumefaciens* were recently documented in thin cell layer explants of *Brassica napus* L. and were partially overcome by replacing agar with agrose in the plant medium. A hypersensitive response was more likely to occur when small or tender leaf tissue was used (cotyledons or young leaves) [13].

There was a large difference in the degree of transformation caused by the three bacterial strains. pTiA6-related plasmids (i.e., those in A6 and A66) have less expression of *vir* genes than pTiBo542 plasmids (i.e., those in strain A281) [7]. This difference may explain the lower percentages of transformation of older leaves by strain A6 and A66 (data not shown) and cotyledons transformed by strain A6 (Fig. 3) compared to strain A281. Interaction between the already high levels of auxin production in tomato cotyledons (inferred from intense root formation on hormone-free medium) (Fig. 1) and strain A66 may result in a higher degree of tumor formation for tomato cotyledons. Cotyledons exposed to strains A6 and A66 exhibited more necrosis per cotyledon as well as a larger number of cotyledons showing necrosis per plate as compared to cotyledons exposed to strain A281. Strain A6 caused more necrosis at lower bacterial concentrations than the other two strains and greater amounts of necrosis in cotyledons of all cultivars than strains A281 and A66 (data not shown).

Old leaves (Leaf 1) were transformed at percentages comparable to cotyledons (with strain A281) and old leaves were transformed to a higher degree than young leaves (Leaf 5) (Fig. 4). These results contrast with those of Armstead & Webb [14] who found that cotyledons of *Lotus corniculatus* were more readily transformed than leaves from seedlings grown in vitro. Young leaves were transformed more frequently than old leaves. However, leaves from old papaya plants were found to be more easily transformed than cotyledons or leaves from young plants [15].

Leaves apparently lost some morphogenic potential present in cotyledons since no shoot formation occurred after transformation of leaves by strains A66 or A281. The shoot-forming potential was greatest for cotyledons of Ohio 7870 (Table 2). It is possible that integration or expression of T-DNA is different in older leaves than in cotyledons or there is a differing host response to expression of the *onc* genes. Cotyledons were the best tissue for transformation of tomato due to higher shoot regeneration potential on regeneration medium and ease of obtaining sterile material.

In conclusion, factors such as plant cultivar, leaf age, necrotic response, bacterial strain, and concentration should be taken into consideration when designing transformation procedures with *A. tumefaciens*. The differences observed in these studies for cultivars and cotyledons versus older leaves could be used to further study expression of *onc* genes or host response to *onc* genes. Engineered, non-disarmed strains based on strain A281 could possibly be used to transfer genes of interest to tomato and regenerate recombinant tomato plants from cotyledons without use of regeneration medium, although regenerated plants would still have to be screened carefully for transformation.

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References

1. McCormick S, Niedermeyer J, Fry J, Barnason A, Horsch R & Fraley R (1986) Leaf disk transformation of cultivated tomato. (*L. esculentum*) using *Agrobacterium tumefaciens*. Plant Cell Rep. 5: 81–84
2. Shahin EA, Sukhapinda K, Simpson RB & Spivey R (1986) Transformation of cultivated tomato by a binary vector in *Agrobacterium rhizogenes*: transgenic plants with

normal phenotypes harbor binary vector T-DNA, but no Ri-plasmid T-DNA. Theor. Appl. Genet. 72: 770–777
3. Fillati JJ, Kiser J, Rose R & Comai L (1987) Efficient transfer of glyphosate tolerance gene into tomato using a binary *Agrobacterium tumefaciens* vector. Biotechnology 5: 726–730
4. Fischhoff DA, Bowdish KS, Perlak FJ, Marrone PG, McCormick, SM, Niedermeyer JG, Duff DA, Kusano-Kretzner K, Mayer EJ, Rochester DE, Rogers SG & Fraley RT (1987) Insect tolerant transgenic tomato plants. Biotechnolgy 5: 807–813
5. Chyi YS, Jorgensen RA, Goldstein D, Tanksley SD & Loaiza-Figueroa F (1986) Locations and stability of *Agrobacterium*-mediated T-DNA insertions in the *Lycopersicon* genome. Mol. Gen. Genet. 204: 64–69
6. Chyi YS & Phillips GC (1987) High efficiency of *Agrobacterium*-mediated transformation of *Lycopersicon* based on conditions favorable for regeneration. Plant Cell Rep. 6: 105–108
7. Engstrom P, Zambryski P, Van Montagu M & Stachel S (1987) Characterization of *Agrobacterium tumefaciens* virulence proteins induced by the plant factor acetosyringone. J. Mol. Biol. 197: 634–645
8. Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497
9. Parsons TJ, Sinkar VP, Stettler RF, Nestor EW & Gordon MP (1986) Transformation of poplar by *Agrobacterium tumefaciens*. Biotechnology 4: 533–536
10. Anscombe FJ (1948) The transformation of poisson, binomial, and negative binomial data. Biometrika 35: 246–254
11. Mize CW & Chun YW (1988) Analyzing treatment means in plant tissue culture research. Plant Cell Tiss. Org. Cult. 13: 201–217
12. Eapen-Kohler SF, Gerdemann M & Schieder O (1987) Cultivar dependence of transformation rates on moth bean after co-cultivation of protoplasts with *Agrobacterium tumefaciens*. Theor. Appl. Genet. 75: 207–210
13. Charest PJ, Holbrook LA, Gabard J, Iyer VN & Miki BL (1988) *Agrobacterium*-mediated transformation of thin cell layer explants from *Brassica napus* L. Theor. Appl. Genet. 75: 438–445
14. Armstead IP & Webb KJ (1987) Effect of age and type of tissue on genetic transformation of *Lotus corniculatus* by *Agrobacterium tumefaciens*. Plant Cell Tiss. Org. Cult. 9: 95–101
15. Pang SZ & Sanford JC (1988) *Agrobacterium*-mediated gene transfer in papaya. J. Am. Soc. Hort. Sci. 113: 287–291

Plant Physiol. (1996) 112: 115–120

Insect Control and Dosage Effects in Transgenic Canola Containing a Synthetic *Bacillus thuringiensis cryIAc* Gene[1]

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Zygotic hypocotyls of canola (*Brassica napus* L.) cv Oscar, cv Westar, and the breeding line UGA188–20B were transformed with a truncated synthetic *Bacillus thuringiensis* insecticidal crystal protein gene (*Bt cryIAc*) under the control of the cauliflower mosaic virus 35S promoter using *Agrobacterium tumefaciens*-mediated transformation. Fifty-seven independently transformed lines were produced, containing 1 to 12 copies of the transgenes. A range of *cry* expressors was produced from 0 to 0.4% Cry as a percentage of total extractable protein. The *Brassica* specialists, the diamondback moth (*Plutella xylostella* L.) and the cabbage looper (*Trichoplusia ni* Hübner), were completely controlled by low-, medium-, and high-expressing lines. Whereas control of the generalist lepidopteran, the corn earworm (*Helicoverpa zea* Boddie), was nearly complete, the other generalist caterpillar tested, the beet armyworm (*Spodoptera exigua* Hübner), showed a dose response that had a negative association between defoliation and *cry* expression. These plants were produced as models for an ecological research assessment of the risk involved in the field release of naturalized transgenic plants harboring a gene (*Bt*) that confers higher relative fitness under herbivore-feeding pressure.

The seeds of a number of *Brassica* L. species are cultivated for the production of oil. Collectively, the oilseed *Brassica* sp., generally referred to as rapeseed, supply more than 13% of the world's supply of edible oils and rank third behind soybean and oil palm in importance. The term "canola" was adopted by the Canadians in 1979 and used to describe oilseed *Brassica* cultivars that produce oils containing less than 2% erucic acid and to describe defatted seed meals with less than 30 μmol g^{-1} of aliphatic glucosinolates. *Brassica napus* L. canola cultivars are currently dominant in U.S. production, although canola-quality *Brassica rapa* (synonymous with *Brassica campestris*) cultivars also exist (Raymer et al., 1990).

Production of canola in the U.S. has grown at a modest rate during the last 10 years from virtually 0 in 1985 to 165,000 ha in 1995 (C. Boynton, U.S. Canola Association, personal communication). As production of canola continues to grow, insect problems are expected to become more serious (Lamb, 1989). This may be particularly true as canola production expands in the southeastern United States and California, where mild winter temperatures are likely to lead to increased herbivory, as compared with the much cooler areas (e.g. Canada), where canola has historically been produced. Ubiquitous lepidopteran *Brassica* specialists, such as the DBM and the CBL, and generalist lepidop- terans, such as the BAW and CEW, may increase in importance where canola is grown in warmer regions (Buntin and Raymer, 1994). This scenario may be especially viable with regard to generalist herbivores, since glucosinolates, a hypothesized antiherbivorant to generalist insects (Giamoustaris and Mithen, 1995), have been bred out of canola-quality rapeseed. Transgenic canola cultivars with insecticidal properties will certainly play a major role in integrated pest management strategies for canola pests (Talekar and Shelton, 1993; Evans and Scarisbrick, 1994).

The objectives for this study were 2-fold. (a) To determine the effect of *Bt* expression in *B. napus* on antibiosis for several lepidopteran insects. Unlike the related *Bt* soybean study (Stewart et al., 1996), in which only few, low-expressing synthetic *Bacillus thuringiensis* insecticidal crystal protein (*Bt cryIAc*) plants were produced, the likelihood of obtaining a wide range of *Bt* expression is greater using a species that is more amenable to genetic transformation, such as *B. napus*. (b) To develop biological tools to test models pertaining to biotechnological risk assessment. It is possible that a plant species, such as *B. napus*, that is able to persist in nonagricultural environments could become more weedy in a transgenic form if the transgene confers an increment of fitness and the plant is naturalized in areas of its cultivation. Thus, we developed the *Bt* canola to ultimately test population-level ecological hypotheses.

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Abbreviations: BAW, beet armyworm (*Spodoptera exigua* Hübner); *Bt*, *Bacillus thuringiensis*; CBL, cabbage looper (*Trichoplusia ni* Hübner); CEW, corn earworm (*Helicoverpa zea* Boddie); DBM, diamondback moth (*Plutella xylostella* L.); *HPH*, hygromycin phosphotransferase gene; HSD, honestly significant difference; MS, Murashige-Skoog; T_1, progeny from primary transgenic plants.

MATERIALS AND METHODS

Vector, Construct, and *Agrobacterium tumefaciens* Strain

We used the same synthetic *Bt cryIAc/HPH* construct as described by Stewart et al. (1996). This was placed in the vector pH602 (Firoozabady et al., 1987), which contains a hygromycin-selectable marker under control of the cauliflower mosaic virus 35S promoter (renamed pH602/Bt; Singset et al., 1996; Fig. 1). pH602/Bt was electroporated into the *Agrobacterium tumefaciens* strain GV3850, a disarmed C58 derivative.

Tissue Culture and Genetic Transformation

The tissue culture and transformation procedures were modified from Mehra-Palta et al. (1991). Seedlings and tissue cultures were maintained in a growth room under cool-white fluorescent lights (30 μE irradiance), 23-h photoperiods, and 25°C. Seeds of *Brassica napus* cv Westar, cv Oscar, and the breeding line UGA188–20B were surface-sterilized in 10% commercial bleach for 20 min and germinated on MS basal medium (Murashige and Skoog, 1962, as modified by Mehra-Palta et al., 1991) containing MS salts, 40 mg L^{-1} $FeNa_2$ EDTA, 100 mg L^{-1} *myo*-inositol, 0.1 mg L^{-1} nicotinic acid, 0.1 mg L^{-1} pyridoxine HCl, 0.02 mg L^{-1} thiamine HCl, 0.4 mg L^{-1} Gly, 30 g L^{-1} Suc, and 0.5% agarose (SeaKem, FMC Products, Rockland, ME). Hypocotyls were excised from 5-d-old seedlings and preconditioned for 24 h on basal medium containing 1 mg L^{-1} 2,4-D. Hypocotyls were treated with an *Agrobacterium* solution (10^8–10^9 cells mL^{-1} in liquid MS basal medium) for 30 min and co-cultivated for 3 d on basal medium with 1 mg L^{-1} 2,4-D. After co-cultivation, the hypocotyl segments were transferred to the same medium containing 500 mg L^{-1} Mefoxin (Merck, West Point, PA) and 10 mg L^{-1} hygromycin to select transformed cells. After 1 week, the hypocotyl segments were transferred to basal medium containing 4 mg L^{-1} 6-benzylaminopurine, 2 mg L^{-1} zeatin, 5 mg L^{-1} silver nitrate, and selection agents at the above rates. Transfers to fresh medium were made every 3 weeks. After shoot formation (in 3–6 weeks), shoots were transferred to basal medium containing 0.05 mg L^{-1} 6-benzylaminopurine plus Mefoxin for shoot elongation. After 2 to 4 weeks, shoots were transferred to basal medium containing 0.1 mg L^{-1} indole butyric acid plus Mefoxin to initiate root formation. Alternatively, root initiation was accomplished by placing shoots rooted in a sand/bark mixture in 2-inch pots subsequent to dipping in Rootone (Rigo, Buckner, KY) containing 0.1% indole butyric acid. After plants were rooted, they were hardened-off and transferred to 35-cm-long × 10-cm-diameter conical plastic pots. Primary transgenic plants were grown in a growth chamber with 500 μE irradiance, 16-h photoperiods, and thermoperiods of 21 and 16°C. Seeds were harvested, and T_1 plants were grown in 400-mL Styrofoam cups under the same conditions as above but under 12-h photoperiods to maintain plants in a vegetative state for the insect bioassays.

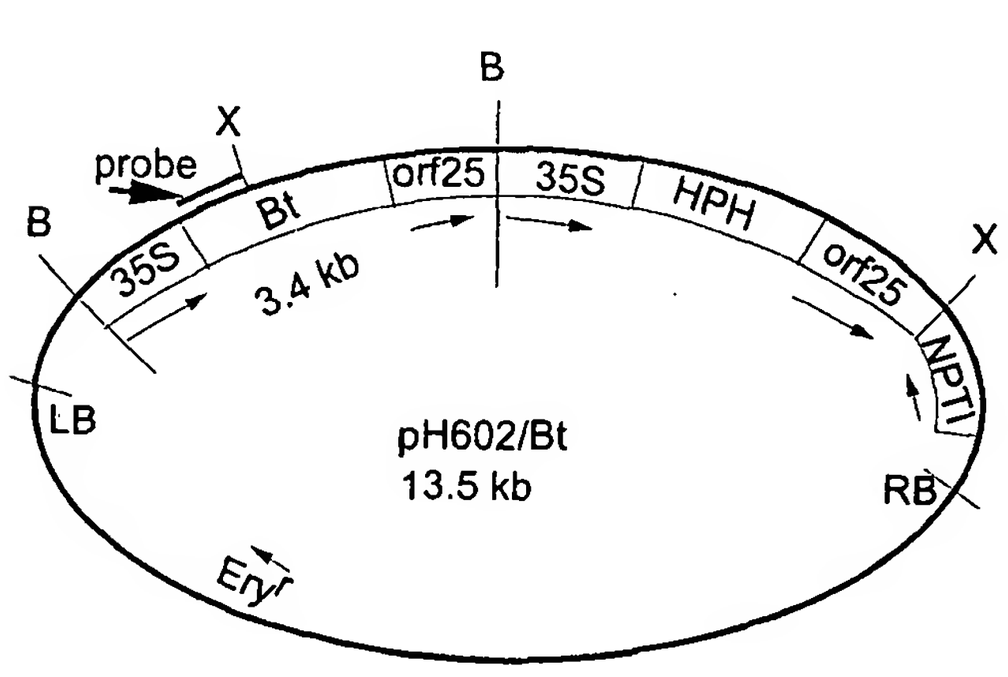

Figure 1. Plasmid pH602/Bt. The *Agrobacterium* transformation vector contains the same *Bt/HPH* construct as reported by Stewart et al. (1996). B, *Bgl*II; X, *Xba*I; LB, left border; RB, right border; orf25, open reading frame 25; Ery^r, erythromycin resistance; NPTI, neomycin phosphotransferase I. The PCR fragment used as a probe within the *cry*-coding region is denoted.

DNA and Protein Blot Analyses

DNA blot analysis and protein blot analysis (Stewart et al., 1996) were used with 10 μg of total protein in each sample lane of the protein gels. In addition, we tested for an association between transgene copy number and expression level using a Spearman rank-correlation test (SAS Institute, 1990).

Insect Bioassays

Detached leaf-insect bioassays were performed on T_1 plants using previously described containers and methods (Parrott et al., 1994). These containers held single, detached leaves and 10 neonate larvae (except for 15 DBM larvae) that were approximately 2 h old. The following insects were tested singularly: DBM, CBL, CEW, and BAW. Plant class was determined by transgenic line (Westar or Oscar) and *Bt* status (±). Five response variables were measured. Percentage of defoliation and insect survivorship data were collected at the end of each 6-d trial. Insect body length, head capsule diameter, and fresh mass were measured after insects were collected and frozen at the end of the trial. The completely random factorial design included eight plant classes, which consisted of different independent T_1 *Bt*-transformants, and T_1 *Bt*-null segregants × eight plants per class (some lines had fewer plants tested) × two replicate leaves per plant = n = 120 to 180 data points for each of the five response variables per each insect tested. Analysis of variances were performed by insect. Multiple comparisons were made using Tukey's HSD (SAS Institute, 1990). We tested for an association between transgene expression and antibiosis response variables by performing Spearman correlations (SAS Institute, 1990).

RESULTS

Genetic Transformation and Primary Transformants

We recovered 57 independently transformed lines of *Bt* canola. Whereas most canola transformation work has been

Table I. *Summary of genetic transformation efficiency of Agrobacterium-mediated transformation of three canola cultivars with a synthetic Bt crylAc gene*

Cultivar	Start[a]	Callus[b]	Shoots[c]	Rooted[d]	Transgenic[e]	Fertile[f]
Oscar	560	161	155	98	40	32
UGA 188-20B	800	199	28	22	7	5
Westar	720	278	82	20	10	9

[a] Number of hypocotyl segments co-incubated in *Agrobacterium* solution. [b] Number of hypocotyl segments producing callus during hygromycin selection. [c] Number of total shoots from callus. [d] Number of rooted shoots, either in vitro or ex vivo. [e] Number of transgenic plants as shown by DNA blot analysis. [f] Number of fertile transgenic plants determined by seed set.

performed using cv Westar, we achieved the highest efficiencies using cv Oscar, in which 7% of hypocotyl segments that were exposed to *Agrobacterium* harboring the *Bt* construct produced transgenic plants (Table I). For this reason, we chose to focus most of our T_1 studies primarily on the recovered Oscar lines.

Transgenes were stably integrated as shown by DNA blot analysis. The mean and median transgene copy numbers were 5.15 and 5, respectively. The mean and median *cry* expressions were 554 and 312 ng mg^{-1}, respectively. We recovered only four transformants with single-copy inserts (9% of fertile transformed lines).

Molecular Analyses of T_1 Transgenic Plants

We germinated 10 to 20 seeds from a range of Oscar (o) and Westar (w) *Bt* expressors for formal analyses. The small number of seeds germinated was insufficient to determine segregation patterns except in the one- or multicopy lines, in which the transgene copies were obviously linked (Fig. 2). Of these lines (namely o52, o56, and o68) segregation patterns were Mendelian (3:1) using a χ^2 test at $P < 0.05$ (Fig. 2). Expression was low (o68, w58; less than 50 ng CryIAc mg^{-1} protein); moderate (o3, o56; about 270 ng CryIAc mg^{-1} protein); moderately high (o52; 586 ng CryIAc mg^{-1} protein); and high (o96, w53; more than 1300 ng CryIAc mg^{-1} protein) (Table II; Fig. 3). Segregating nontransgenics were used for negative controls (the "no" classification). Sample sizes for Westar lines were small because of resource limitations.

Insect Bioassays Performed on T_1 Transgenic Plants

The Cry-susceptible *Brassica* specialists DBM and CBL, assayed against transgenic plants using detached leaf-infestation assays, were completely inhibited by transgenic plants, regardless of *cry* expression level. All DBM and CBL were killed by transgenic plants except for w58, a low-expressing Westar line in which insect survivorship and defoliation were less than 5% than that of nontransgenic. The other low-expressing line (o68) also allowed a few CBL larvae to survive during the assay.

Similarly, the results of trials using the generalist CEW showed significant differences ($P = 0.05$) only between nontransgenics and transgenics. However, on both low-expressing transgenic lines (o68 and w58) and moderate expressors (o3 and o56) a few larvae survived the assay (Table III). There were no significant differences in the growth characteristics of those insects that survived (Table III). There were slight amounts of defoliation, except in the o56 line, in which no defoliation was noted (Table III).

In contrast, there was a noticeable gradation of insect survivorship, defoliation, and insect growth among transgenics in the trials using Cry-tolerant BAW (Table IV). Here, both analysis of variance and Spearman correlations showed a negative association between insect survivorship, growth, defoliation, and Cry expression. Insect survivorship was more than 3 times higher on nontransgenic plants as compared with a high-expressing transgenic line. Also, nontransgenic plants suffered more than 25 times the defoliation (Table IV). Correlation analysis showed a good logarithmic fit between expression and survivorship ($r = -0.51$ for nontransformed data; $r = -0.73$ for log transformed data) and defoliation ($r = -0.52$ for nontransformed data; $r = -0.74$ for log transformed data) (Fig. 4). Correlation analysis also showed a significant negative association between expression and surviving insect cap-

Figure 2. DNA blot analysis of four T_1 transgenic lines. Ten micrograms of genomic DNA and 50 pg of pH602/Bt (Bt) were digested with *Xba*I, electrophoresed in a 1% agarose gel, blotted onto a nylon filter, and hybridized to a ^{32}P-labeled *cry*-specific probe. M, 1-kb DNA ladder (GIBCO-BRL). Genomic DNA was isolated from leaves of T_1 plants (1-copy *Bt* lines o56 and o68 and multicopy lines o52 and o96). The copies are obviously linked in o52. See Table II for line characteristics.

Table II. *Summary of T_1 Bt canola molecular and expression data*

Different letters indicate significant differences using Tukey's HSD at $\alpha = 0.05$.

Line	Transgene Copy No.	n^a	CrylAc Synthesis
			ng mg^{-1}
o3	6	5	269 ± 86 a
o52	2	7	586 ± 356 ab
o56	1	7	267 ± 130 a
o68	1	6	5 ± 0 a
o96	4	5	1379 ± 341 c
w53	2	3	1463 ± 271 c
w58	3	3	40 ± 10 a

[a] n = number of 2-week-old leaves/plants sampled for immunoblot analysis.

sule head diameter ($r = -0.35$), body length ($r = -0.54$), and weight ($r = -0.45$). In all cases $P < 0.0001$, except for the insect head capsule diameter, for which $P = 0.004$.

DISCUSSION

Transgenic Plants

The transformation method used was effective in producing a large number of stably transformed canola plant lines. Oscar, a blackleg (*Leptosphaeria maculans*)-resistant Australian cultivar recently introduced into the southeastern United States for use in blackleg-infested areas (Raymer et al., 1995), had the highest transformation efficiencies. This is the first report, to our knowledge, of transgenic Oscar. The high relative transformation frequencies (compared to that of Westar) coupled with the blackleg resistance makes Oscar a superior candidate for canola transformation studies.

The maximum level of *Bt* expression reported here (0.4%, Cry production as a percentage of total extractable protein) is among the highest reported in the literature, owing to at least some degree to an efficient recoding of the endotoxin gene and the large number of independent transformants produced. For example, researchers who have transformed plants with synthetic *Bt* genes under the control of a constitutive promoter have recovered plants expressing a wide range of maximums: 0.025 and 0.1% of CryIIIA in potato (Adang et al., 1993; Perlak et al., 1993); 0.03% of CryIAb in tobacco (Perlak et al., 1991); 0.05% of CryIAb in rice (Fujimoto et al., 1993); and 0.4% of CryIAb in maize (Koziel et al., 1993). A recently published paper by McBride et al. (1995) demonstrated an alternative to transgenics with synthetic *Bt* genes by engineering a native *CrylAc* into tobacco chloroplasts, yielding transgenic plants with 3 to 5% Cry. Although the expression level was very high and no gene reconstruction was needed, transgenic chloroplasts will continue to segregate, providing the possibility of irregular inheritance.

Insect Control

We tested a suite of lepidopterans ranging in Cry susceptibility, including both *Brassica* specialists and polyphagous species. From most susceptible to least susceptible to Cry toxins, the insects rank CBL > DBM = CEW > BAW (MacIntosh et al., 1990; Moar et al., 1990; Tabashnik et al., 1994). BAW, along with other *Spodoptera* sp., are known to be tolerant to *Bt* toxins (Moar et al., 1990). Feeding was prevented by three of the species by even the lowest expressors, whereas the generalist BAW, although negatively affected by high doses of endotoxin, survived on all transgenics (Table IV). The gradation of *cry* expressors provided an opportunity to test the effect of expression level on antibiosis of a *Bt*-tolerant insect species (BAW). Dose effects have only been rarely examined and documented using *Bt* transgenics. A priori, one would intuitively expect a positive correlation between *Bt* dose (expression) and antibiosis. Adang et al. (1993) found a positive association between *cryIIIA* expression and antibiosis of Colorado potato beetle on transgenic potato. Other researchers who have generated large numbers of independent transformants have not reported this association (Perlak et al., 1991; Koziel et al., 1993). However, in these studies, either the research involved insect species that are very sensitive to *Bt* toxin, such that all individuals were killed regardless of expression, or the experimental design did not test explicitly for the association of expression and antibiosis. Dose effects will likely play an important role in two ecological problems: the evolution of insect resistance to *Bt* and the evolution of weedier *Brassica*.

Insect Resistance to *Bt*

Perhaps the greatest potential problem with the commercialization and environmental release of *Bt* transgenic plants is that of the evolution of insect resistance to *Bt*. Wild populations of DBM have been collected that show high levels of resistance to CryI toxins (Tabashnik, 1994). Perhaps even more disconcerting is the fact that three different *Bt*-resistant colonies of *Heliothis virescens* have been collected, and all three lines have mapped to three different genetic loci on a *H. virescens* genetic linkage map (Heckel, 1994). Thus, with no strategy in place for manag-

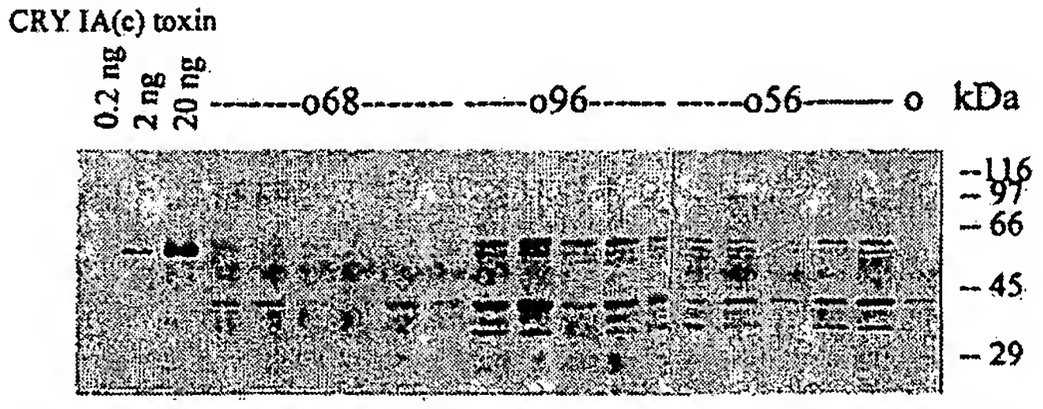

Figure 3. T_1 Bt CrylAc protein blot analysis. Immunostained blot shows typical progeny expression from low (o68), high (o96), and medium (o56) *cry* expressors. See Table II for line characteristics. o, Untransformed Oscar. Densitometric measurements were performed on the two top bands of each lane. These two bands represent the size anticipated (that of the standard) and a slightly larger protein. The three lower, specifically immunostained bands were assumed not to be biologically active and therefore not used in the estimate of expression.

Table III. *Summary of detached-leaf insect bioassays of CEW, a generalist lepidopteran that is somewhat tolerant to CrylAc*

The means and SDs are presented. Different letters within columns denote significant differences at P = 0.05 (Tukey's HSD). * denotes no insect survivors.

Line	*n*	Live Larvae	Head Capsule	Body Length	Larval Weight	Defoliation
			mm	*mm*	*mg*	%
no	14	4.71 ± 2.23 a	0.75 ± 0.23 a	6.38 ± 2.26 a	6.12 ± 5.33 a	26.8 ± 26.6 a
o3	18	0.22 ± 0.55 b	0.32 ± 0 a	2.70 ± 0.52 a	0.50 ± 0.26 a	0.28 ± 0.96 b
o52	14	0 b	0.30 ± 0 a	4.00 ± 0 a	0.20 ± 0 a	0 b
o56	16	0.06 ± 0.25 b	0.30 ± 0 a	2.40 ± 0 a	0.20 ± 0 a	0 b
o68	14	0.21 ± 0.43 b	0.43 ± 0.12 a	3.37 ± 1.51 a	4.33 ± 1.0 a	0.36 ± 0.63 b
o96	16	0 b	*	*	*	0 b
w53	5	0 b	*	*	*	0 b
w58	4	0.50 ± 0.58 b	0.39 ± 0.13 b	3.15 ± 0.21 b	0.60 ± 0.14 b	0.50 ± 0.58 b

ing resistance, some insect populations will evolve resistance very quickly through strong selective pressures and the presence of multiple resistance genes. Although various strategies to manage resistance have been proposed, there is little empirical data showing that one strategy is superior to another. However, to manage *Bt* resistance, mechanisms of insect resistance must be elucidated to make predictive models of insect evolution. Transgenic plants with a gradation of *cry* expression provide research tools allowing researchers to select for *Bt*-resistant insects in controlled settings for the purpose of studying modes and dynamics of the evolution of *Bt* resistance. Furthermore, Cry-tolerant insect species such as BAW, which may be affected by Cry toxins at relatively high doses, could be useful as part of that system to study dose effects of *Bt* toxins.

Bt Transgenes May Increase Weediness of *Brassica*

Another risk in deploying *Bt* transgenic plants that has been discussed very little is that of increasing the fitness of the host. For insecticidal transgenic plants, and for any transgenic plants engineered with a gene that may confer an increment of fitness to its host, there is the possibilitythat host plants may become more weedy. Theoretically, this scenario could occur under only two conditions: (a) the host plant is already naturalized in a nonagricultural environment and/or it is sexually compatible with a species that is naturalized, and (b) there is selection pressure in the nonagricultural environment. The combination of *Bt* and canola fulfills these requirements, thus making it a good model to test some basic hypotheses. We will be using *Bt* canola in a field study to investigate the effect that insect-feeding pressure may have on population replacement of nontransgenic plants with transgenic plants with *Bt*. Such a study will provide data that can be used to assess the risk involved in deploying naturalized transgenic plants with a transgene conferring fitness to its host. In addition, and perhaps most important, it is becoming clear that transgenes in canola will be introgressed rapidly into weedy relatives such as *B. campestris* (synonymous with *B. rapa*) and *Raphanus raphinistrum* (Jørgensen and Andersen, 1994; Scheffler and Dale, 1994; Mikkelsen et al., 1996). Such genes, such as *Bt cryIAc*, at threshold levels of insect-feeding pressure, will likely increase the relative fitness of its host. However, it is not known whether these effects will translate into increased weediness. Additional research is needed to address these concerns, and it seems that the *Bt* canola produced in this study will be a useful tool to this end.

Table IV. *Summary of detached-leaf insect bioassays of BAW, a generalist lepidopteran that is very tolerant to CrylAc*

The means and SDs are presented. Different letters within columns denote significant differences at P = 0.05 (Tukey's HSD). Feeding data are from bioassays of BAW, a generalist lepidopteran that is resistant to CrylAc.

Line	*n*	Live Larvae	Head Capsule	Body Length	Larval Weight	Defoliation
			mm	*mm*	*mg*	%
no	12	6.50 ± 1.57 a	0.97 ± 0.14 a	8.38 ± 1.57 a	13.4 ± 6.1 a	70.8 ± 16.5 a
o3	13	3.62 ± 1.66 bc	0.45 ± 0.11 b	3.52 ± 1.06 bcd	1.55 ± 1.39 b	5.85 ± 6.67 c
o52	12	2.00 ± 1.60 c	0.36 ± 0.09 b	2.54 ± 0.74 e	0.49 ± 0.32 b	2.75 ± 1.76 c
o56	14	3.50 ± 1.79 bc	0.41 ± 0.08 b	3.01 ± 0.62 d	0.88 ± 0.55 b	7.21 ± 6.57 c
o68	10	4.50 ± 2.92 ab	0.57 ± 0.27 b	4.81 ± 2.33 bc	4.75 ± 5.67 b	29.2 ± 24.7 b
o96	14	3.07 ± 1.94 bc	0.50 ± 0.51 b	2.56 ± 0.53 e	0.53 ± 0.35 b	3.86 ± 3.78 c
w53	6	4.00 ± 3.03 bc	0.37 ± 0.10 b	2.72 ± 0.82 e	0.46 ± 0.27 b	6.17 ± 6.85 c
w58	4	6.00 ± 2.83 ab	0.63 ± 0.18 a	5.21 ± 2.28 b	4.79 ± 3.66 b	40.8 ± 29.0 b

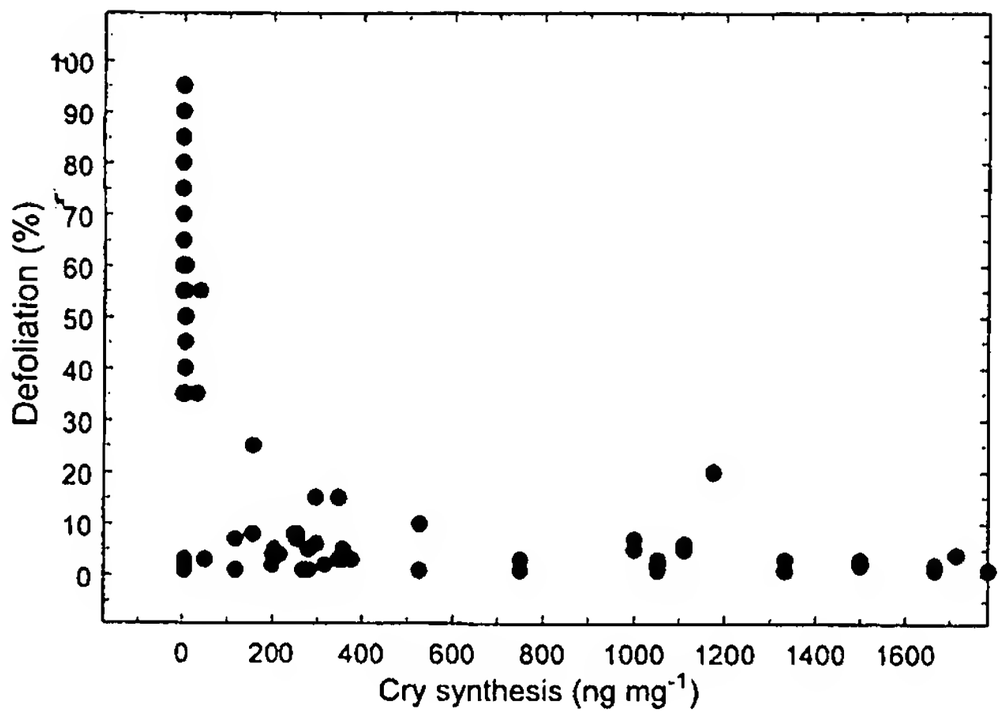

Figure 4. Defoliation versus Cry synthesis. Detached-leaf bioassays were performed in which 10 BAW were applied to each 2-week-old leaf. Defoliation after 6 d was negatively associated with *cry* expression at $P > 0.0001$.

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LITERATURE CITED

Adang MJ, Brody MS, Cardineau G, Eagan N, Roush RT, Shewmaker CK, Jones A, Oakes JV, McBride KE (1993) The construction and expression of a *Bacillus thuringiensis cryIIIA* gene in protoplasts and potato plants. Plant Mol Biol **21:** 1131–1145

Buntin GD, Raymer PL (1994) Pest status of aphids and other insects in winter canola in Georgia. J Econ Entomol **87:** 1097–1104

Evans KA, Scarisbrick DH (1994) Integrated insect pest management in oilseed rape crops in Europe. Crop Prot **13:** 403–412

Firoozabady E, DeBoer DL, Merlo DJ, Halk EL, Amerson LN, Rashka KE, Murray EE (1987) Transformation of cotton (*Gossypium hirsutum* L.) by *Agrobacterium tumefaciens* and regeneration of transgenic plants. Plant Mol Biol **10:** 105–116

Fujimoto H, Itoh K, Yamamoto M, Kyozuka J, Shimamoto K (1993) Insect resistant rice generated by introduction of a modified δ-endotoxin gene of *Bacillus thuringiensis*. Biotechnology **11:** 1151–1155

Giamoustaris A, Mithen R (1995) The effect of modifying the glucosinolate content of leaves of oilseed rape (*Brassica napus* ssp. *oleifera*) on its interaction with specialist and generalist pests. Ann Appl Biol **126:** 347–363

Heckel DG (1994) The complex genetic-basis of resistance to *Bacillus thuringiensis* toxin in insects. Biocontrol Sci Technol **4:** 405–417

Jørgensen RB, Andersen B (1994) Spontaneous hybridization between oilseed rape (*Brassica napus*) and weedy *B. campestris* (Brassicaceae): a risk of growing genetically modified oilseed rape. Am J Bot **81:** 1620–1626

Koziel MG, Beland GL, Bowman C, Carozzi NB, Crenshaw R, Crossland L, Dawson J, Desai N, Hill M, Kadwell S, Launis K, Lewis K, Maddox D, McPherson K, Meghji MR, Mevlin E, Rhodes R, Warren GW, Wright M, Euola SV (1993) Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. Biotechnology **11:** 194–200

Lamb RJ (1989) Entomology of oilseed *Brassica* crops. Annu Rev Entomol **34:** 211–229

MacIntosh SC, Stone TB, Sims SR, Hurst JT, Greenplate JT, Marrone PG, Perlak FJ, Fischoff DA, Fuchs RL (1990) Specificity and efficacy of purified *Bacillus thuringiensis* proteins against agronomically important insects. J Invertebr Pathol **56:** 258–266

McBride KE, Svad Z, Schaaf DJ, Hogan PS, Stalker DM, Maliga P (1995) Amplification of a chimeric *Bacillus thuringiensis* gene in chloroplasts leads to an extraordinary level of insecticidal protein in tobacco. Biotechnology **13:** 362–365

Mehra-Palta A, Graves A, Kraemer M, Hill K, Eagan N (1991) Genetic transformation of *Brassica napus* and *Brassica rapa*. *In* Anonymous Rapeseed in a Changing World. du Groupe Consultif International de Recherche sur le Colza Congress, Saskatoon, Saskatchewan, Canada, pp 1108–1115

Mikkelsen TR, Andersen B, Jørgensen RB (1996) The risk of crop transgene spread. Nature **380:** 31

Moar MJ, Moar WJ, Masson L, Brousseau R, Trumble JT (1990) Toxicity to *Spodoptera exigua* and *Trichoplusia ni* of individual P1 protoxins and sporulating cultures of *Bacillus thuringiensis* subsp. *kurstaki* HD-1 and NRD-12. Appl Environ Microbiol **56:** 2480–2483

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15:** 473–497

Parrott WA, All JN, Adang MJ, Bailey MA, Boerma HR, Stewart CN Jr (1994) Recovery and evaluation of soybean (*Glycine max* [L.] Merr.) plants transgenic for a *Bacillus thuringiensis* var. *kurstaki* insecticidal gene. In Vitro Cell Dev Biol **30P:** 144–149

Perlak FJ, Fuchs RL, Dean DA, McPherson SL, Fischhoff DA (1991) Modification of the coding sequence enhances plant expression of insect control protein genes. Proc Natl Acad Sci USA **88:** 3324–3328

Perlak FJ, Stone TB, Muskopf YM, Petersen LJ, Parker GB, McPherson SA, Wyman J, Love S, Reed G, Biever D, Fischoff DA (1993) Genetically improved potatoes: protection from damage by Colorado potato beetles. Plant Mol Biol **22:** 313–321

Raymer PL, Auld DL, Mahler KA (1990) Agronomy of canola in the United States. *In* F Shahidi, ed, Canola and Rapeseed: Production, Chemistry, Nutrition and Processing Technology. Van Nostrand Reinhold, New York, pp 25–35

Raymer PL, Day JL, Bennet RB, Coy AE (1995) 1994–1995 canola performance tests. Georgia agriculture experimental station research report, Ed 635. University of Georgia, Athens, pp 1–11

SAS Institute (1990) SAS/STAT User's Guide, version 6. SAS Institute, Cary, NC

Scheffler JA, Dale PJ (1994) Opportunities for gene transfer from transgenic oilseed rape (*Brassica napus*) to related species. Transgen Res **3:** 263–278

Shahidi F (1990) Rapeseed and canola: global production and distribution. *In* F Shahidi, ed, Canola and Rapeseed: Production, Chemistry, Nutrition and Processing Technology. Van Nostrand Reinhold, New York, pp 3–13

Singset C, Adang MJ, Lynch RE, Anderson WF, Wang A, Cardineau G, Ozias-Akins P (1996) Expression of *Bacillus thuringiensis cryIA(c)* gene in transgenic peanut plants and its efficacy against lesser cornstalk borer. Transgen Res (in press)

Stewart CN Jr, Adang MJ, All JN, Boerma HR, Cardineau G, Tucker D, Parrott WA (1996) Genetic transformation, recovery, and characterization of fertile soybean transgenic for a synthetic *Bacillus thuringiensis cryIAc* gene. Plant Physiol **112:** 121–129

Tabashnik BE (1994) Evolution of resistance to *Bacillus thuringiensis*. Annu Rev Entomol **39:** 47–79

Tabashnik BE, Finson N, Groeters FR, Moar WJ, Johnson MW, Luo K, Adang MJ (1994) Reversal of resistance to *Bacillus thuringiensis* in *Plutella xylostella*. Proc Natl Acad Sci USA **91:** 4120–4124

Talekar NS, Shelton AM (1993) Biology, ecology, and management of the diamondback moth. Annu Rev Entomol **38:** 275–301